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Evaluation of PCR-Based Methods for Rapid, Accurate Detection and Monitoring of Verticillium Dahliae in Woody Hosts by Real-Time Polymerase Chain Reaction

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EVALUATION OF PCR-BASED METHODS FOR RAPID, ACCURATE DETECTION AND MONITORING OF VERTICILLIUM DAHLIAE IN WOODY HOSTS BY REAL-TIME POLYMERASE CHAIN REACTION

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food, and Environment at the University of Kentucky

By

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ABSTRACT OF THESIS

EVALUATION OF PCR-BASED METHODS FOR RAPID, ACCURATE DETECTION AND MONITORING OF *VERTICILLIUM DAHLIAE* IN WOODY HOSTS BY REAL-TIME POLYMERASE CHAIN REACTION

Verticillium wilt, caused by *Verticillium dahliae* Kleb, is one of the most economically important diseases of woody hosts such as ash (*Fraxinus* spp.), sugar maple (*Acer saccharum*), and redbud (*Cercis canadensis*). The causal agent has a broad host range, including not only woody hosts but also important vegetable and field crops, and it is distributed worldwide. Diagnosis of *V. dahliae* in infected woody hosts is often based on the occurrence of vascular discoloration and time-consuming isolation. However, not all woody hosts exhibit vascular discoloration symptoms, and not all vascular discoloration symptoms are due to infection by *V. dahliae*. In this study, real-time PCR-based assays were evaluated and employed for rapid and accurate detection of *V. dahliae* in different woody hosts. DNA was extracted in large quantities from presumptively infected woody hosts by collecting drill-press shavings from sample tissue, bead-beating, and extracting using a CTAB method. Six published primer sets were evaluated against genomic DNA of *V. dahliae* as well as selected negative controls, and two sets (VertBt-F/VertBt-R and VDS1/VDS2) showed promise for further evaluation using DNA extracts from field samples. The VertBt primers amplified a species-specific 115-bp fragment of the expected size, while the VDS primers amplified the expected specific 540-bp fragment. However, the VertBt primer set exhibited higher sensitivity in detection of *V. dahliae* even in asymptomatic trees. The PCR-based methods developed here could be used as rapid tools for pathogen detecting and monitoring, thus informing plant pathogen management decisions.

KEYWORDS: *Verticillium dahliae*, Verticillium wilt, real-time PCR, woody plants.

Baker Aljawasim

Apr 8th 2014

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To my family, my father, my mother, my friends, and especially Higher Committee for Education Development in Iraq (HCED)

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CHAPTER ONE: LITERATURE REVIEW

I. Verticillium Wilt

Verticillium wilt is one of the most serious and economically important diseases on a broad range of host plants (Bhat & Subbarao, 1999). The principal causal agents of Verticillium wilt are the soil-borne plant pathogens *Verticillium dahliae* Kleb and *Verticillium albo-atrum*, which are distributed throughout the world (Chen, 1994; Pegg & Brady, 2002; Soesanto, 2000). The genus name, *Verticillium,* is derived from a morphological character, the "verticillate" (whorled) arrangement of phialides on the conidiophore (Inderbitzin, Bostock et al., 2011b). Verticillium wilt affects various hosts including trees, shrubs, herbaceous ornamentals, and economically important vegetable and field crops not only in North America, but throughout the world (Bhat & Subbarao, 1999; Hiemstra, 1995; Inderbitzin, Bostock et al., 2011a; Maruthachalam, Atallah et al., 2010; Pegg & Brady, 2002; Sinclair & Lyon, 2005). The disease has been reported in more than 120 species in 35 genera of widely unrelated families in 18 orders (Ash, 1999). Lists of woody hosts have been published in literature reviews (Ash, 1993, 2001; Engelbrecht & Gleason, 2006; Hiemstra, 1995). At least 56 tree and shrub species were listed as hosts for *V. dahliae* by Himelick in 1969, and eight shade tree genera were found most susceptible to *V. dahliae*, including maple (*Acer*), elm (*Ulmus*), ash (*Fraxinus*), catalpa (*Catalpa*), tulip tree

(*Liriodendron*), magnolia (*Magnolia*), redbud (*Cercis*), and Russian olive (*Elaegnus*) (Mace, Bell et al., 1981).

V. dahliae causes billions of dollars of damage annually to a variety of agricultural crops in many parts of the world such as vegetables, fiber crops, fruit and nut trees, legumes, forest trees, and woody and herbaceous ornamentals (Bhat & Subbarao, 1999; Chen, 1994; Klosterman, Atallah et al., 2009; Larkin, Honeycutt et al., 2011; Pegg & Brady, 2002). The estimated yield losses caused by Verticillium wilt on different crops were between 0.31 to 4.40% per annum in the United States during 1952-2008 (Parkhi & Kumar, 2010). In nursery stock, *V. dahliae* causes extensive losses because the infected plants must be destroyed. Also, the disease causes extreme damage on valuable trees in the landscape, because trees infected with *V. dahliae* often must be replaced (Smith, 1976).

History of Verticillium Wilt

In the past, Verticillium wilt diseases have been referred to as black heart, tracheomycosis, hadromycosis (in stone fruits), blue stem (in bush fruits), and true wilt (Parker, 1959). In the United States, the disease was first reported near Claverack, NY in 1914 on maple trees (species not given) of all age classes (Smith, 1976). Two years later, Norway (*Acer platanoides*), sugar (*A. saccharum*), and red maples trees (*A. rubrum*) were inoculated successfully with *V. dahliae* (Zimm, 1918). Verticillium wilt disease was reported on green (*Fraxinus pennsylvanica*) and white ash (*F. Americana*) in Colorado for first time

at 1950 (Ash, 2001). Between 1954 and 1955, a field survey of Verticillium wilt was conducted in New Jersey; Dochinger reported that the disease was most widespread in silver maple (*Acer saccharinum*) (8% of trees infected), followed by Norway maple (2.8% of trees infected), red maple (1.4% of trees infected), and sugar maple (1% of trees infected) (Mace et al., 1981). Between 1939 and 1959, Verticillium wilt caused death in maple tree more than all other diseases (Smith, 1976). Losses due to Verticillium wilt disease on Norway maples reached 85% in an Oregon nursery (Pirone, Dodge et al., 1960). About 20% of all maple trees were lost in Indianapolis between 1969 and 1972 due to infection by *V. dahliae* (Mace et al., 1981). Red maple seedlings were grown from seed brought from six states (Arkansas, Illinois, Minnesota, New Jersey, Ohio and Pennsylvania), and stem-inoculated with a mixture of microsclerotia and conidia from isolates obtained from five different sources; the researchers found that the most resistance seedlings were those obtained from Arkansas and Illinois, whereas the most susceptible seedlings were those from Minnesota and Pennsylvania. The resistance exhibited in Arkansas and Illinois seedlings was referred to as 'tolerance' due to the ability to re-isolate of *V. dahliae* from these seedlings (Hoitink, Sydnor et al., 1979; Pegg & Brady, 2002). On several cultivars of green and white ash in Pacific Northwest nurseries, Verticillium wilt disease was noted in 1996, and on white ash in Wisconsin *V. dahliae* was reported in 1994 (Mol, 1995).

Classification of *V. dahliae*

The genus of *Verticillium* was erected by Nees von Esenbeckin in 1816 based on unique morphologically characters, and approximately 190 species have since been described under this genus (Inderbitzin et al., 2011b; Isaac, 1967; Schnathorst, 1981; Zare, Gams et al., 2004). *V. dahliae* is classified in the fungal kingdom as follows: Super Kingdom: Eukaryota, Kingdom: Fungi, Subkingdom: Dikarya, Phylum: Ascomycota, Subphylum: Pezizomycotina, Class: Sordariomycetes, Subclass: Hypocreomycetidae, Order: Phyllachorales, Family: Plectosphaerellaceae, Genus: Verticillium, and Species: dahliae (Fradin & Thomma, 2006; Schoch, Sung et al., 2009).

Much controversy has surrounded the naming and identifying of the wilt-inducing species of *Verticillium*, and some of these controversies are not entirely resolved even now. Isaac (1949, 1967) provided some details about the history of the debate. For example, another *Verticillium* species, *Verticillium albo-atrum*, was described first as the causal agent of Verticillium wilt before *V. dahliae* (Isaac, 1949). *V. dahliae* and *V. albo-atrum* both stand out as the most important members of this genus in agricultural production and in treatment in the scientific literature. *V. albo-atrum* was described by Reinke and Berthold (1879) as a fungus having dark brown to black resting mycelium, forming mycelial masses by pressing together of contiguous hyphae. The cellular or mycelial masses were called first ‘Dauermycelien’, ‘Sklerotien’, or ‘Zellhauf’ (Pegg & Brady, 2002). *V.*

dahliae was first isolated in 1913 by Klebahn from *Dahlia* sp., and classified under the species *Verticillium albo-atrum* in older literature. At that time, both were considered to be the same species; it was thought that the same fungus produced both microsclerotia and dark melanized hyphae. In the late 1970s, *V. dahliae* was described as a species distinct from *V. albo-atrum*, by Reinke and Berthold (Goud, Termorshuizen et al., 2003). The most important characters for the separation between *V. dahliae* and *V. albo-atrum* as biologically distinct species are difference in growth rate at different temperatures, and the production of survival microsclerotia (Ms) in *V. dahliae* vs. the production in *V. albo-atrum* of brown, pigmented hyphae described as 'Dauermycelien' (Dm), translated to 'resting mycelium' by Isaac (Goud et al., 2003; Isaac, 1949).

A new taxonomic system for the classification of *Verticillium* species was established by Patrik Inderbitzin and Richard M. Bostock in 2011, using phylogenetic analyses, morphological investigations and comparisons to herbarium material. Ten species of *Verticillium*, including five previously undescribed species, were recognized in this system. Partial sequences of an *ACTIN CODING GENE* (*ACT*), *RIBOSOMAL INTERNAL TRANSCRIBED SPACER REGION* (*ITS*), *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GPD*), *TRYPTOPHAN SYNTHASE* (*TS*), and *ELONGATION FACTOR 1-ALPHA* (*EF*) were used to establish phylogenetic relationships among ten species of *Verticillium*. In the phylogenetic analyses, *Verticillium* species were divided into two major groups, designated as Clade

Flavexudans and Clade Flaxnonexudans (Inderbitzin et al., 2011a). The Clade Flavexudans was so named for the production of yellow-pigmented hyphae, and the Clade Flaxnonexudans was so named due to the absence of yellow-pigmented hyphae in this group. Species classified under Clade Flavexudans included *V. albo-atrum* and *Verticillium tricorpus* as well as the new species *Verticillium zaregamsianum*, *Verticillium isaacii* and *Verticillium klebahnii*, of which the latter two were morphologically indistinguishable from *V. tricorpus* but differed in pathogenicity. Species classified under Clade Flavnonexudans included *Verticillium nubilum*, *V. dahliae* and *Verticillium longisporum*, as well as the two new species *Verticillium alfalfae* and *Verticillium nonalfalfae*, which are related to *V. albo-atrum* morphologically (Inderbitzin et al., 2011a).

Morphological and Anatomical Characters

In most *Verticillium* species the hyphae are haploid. The cells in *V. dahliae, V. albo-atrum,* and other *Verticillium* species are monokaryotic, but the hyphal tip may be multinucleate (Schnathorst, 1981). On PDA media, colonies of *V. dahliae* after 10 days in the dark at the room temperature are commonly 4–6 cm in diameter, white at first, later darkening due to the formation of microsclerotia (Inderbitzin et al., 2011b). *V. dahliae* has a vegetative mycelium which is hyaline and septate; nuclei have not been reported to traverse the septal pore (Typas & Heale, 1976). The extension of the hyphae in *V. dahliae* requires the availability of some growth compounds such as diffusible morphogenic

factors (DMFs) which inhibit growth elongation and induce lateral branching (Brandt, 1966). The growth of hyphae is greater under dark conditions because light will inhibit the production of DMFs in culture (Brandt, 1966). Lateral branching may help in conidiogenesis, or may favor anastomosis with other hyphae (Pegg & Brady, 2002). Because *V. dahliae* belongs to a group of fungi which do not produce a sexual stage, anastomosis is the only means by which genetic recombination occurs within the species. Conidia (phialospores) are hyaline, smooth-walled, nonseptate, cylindrical with rounded to oval species and usually single-celled, and they are formed in clusters in a mucilaginous slime on elongated conidiogenous cells called phialides, which are borne in whorls around each conidiophore (Inderbitzin et al., 2011b). The phialides produce a massive number of conidia (Pegg & Brady, 2002).

Morphological features of microsclerotia of *V. dahliae* have been studied after 12 days of inoculation on WA-p medium, described by Inderbitzin and Bostock (2011). They showed that the microsclerotia are rounded to elongated or irregular in shape, composed of rounded, brown-pigmented cells up to 13 mm in diameter, and measuring between 25–100 µm in diameter, but that the aggregates of microsclerotia can measure up to 200 µm in diameter (Inderbitzin et al., 2011b). Microsclerotia are formed by dense aggregates of darkly pigmented, thick-walled hyphal cells, which contain a dark (melanized) outer rind of several cell layers to shield the inner portion (medulla), and they develop via swelling of the hyphae and septation of the cells that enlarge and produce lateral cells (Fradin

& Thomma, 2006). Microsclerotia are typically most abundant in the top 10 cm of the soil, and may be nearly undetectable at a depth of 40 cm (Jordan, 1971).

Disease Cycle

Information on the disease cycle of *V. dahliae* in agricultural crops is extensive, but it is limited for tree species. Therefore, the disease cycle presented here was described based on results obtained from a wide range of crops. The causal agent of Verticillium wilt on woody hosts, *V. dahliae,* generally causes monocyclic disease, which mean the pathogen produces only one cycle of inoculum production and infection during a growing season. However, sometimes *V. dahliae* may cause limited secondary infection by production of conidia on plant tissues infected in the current season, which become airborne and contribute to spread of the pathogen (Fradin & Thomma, 2006). *V. dahliae* survives in soil as microsclerotia for more than 14 years in the absence of a host and it can survive in dried culture at room temperature for 13 years, whereas *V. albo-atrum* can survive in dried culture from 2 to 5 years (Wilhelm 1955, Klosterman, Atallah et al. 2009). Microsclerotia are produced within the residue of infected hosts, and they are incorporated into the soil due to the decomposition of the infected host tissue. More than 300 microsclerotia were formed per leaf when partially disintegrated or green olive leaves, fallen from tree infected with *V. dahliae*, were buried within the top 6 cm of moist soil under drip-irrigated trees (Jimenez-Diaz, Cirulli et al., 2012). The population of *V. dahliae* microsclerotia

in the soil may be increased and replenished as a result of limited saprophytic growth of the pathogen in senescing plants or necrotic tissues (Fradin & Thomma, 2006; Neumann & Dobinson, 2003). Microsclerotia are most numerous in the upper layer of the soil, and they can be dispersed from one field to another by wind-blown leaves from infected hosts, dust storms, runoff from irrigation, and rain water. *V. dahliae* can be transmitted within or among nurseries by using infected scions, buds, and rootstock or during pruning (Sinclair & Lyon, 2005).

Many factors can stimulate microsclerotia to germinate in soil, such as root exudates and release of nutrients within the soil, even from roots that are immune to vascular infection (Hiemstra & Harris, 1998). The chance of establishing a successful infection by microsclerotial germination is increased due to the ability of each individual cell in microsclerotia to germinate once, which allows microsclerotia to germinate multiple times (Fradin & Thomma, 2006). Germinated hyphae emerging from microsclerotia can traverse a limited distance of soil in response to root exudates or released nutrients. The sphere of influence of a nutrient gradient from roots on microsclerotia was calculated to be approximately 100 µm (Huisman, 1982). Hyphae emerging from microsclerotia of *V. dahliae* can enter the root predominantly through micro- or macro- injuries, and they can penetrate the root at sites of emergence of lateral rootlets as well, but not through intact epidermal cells (Fradin & Thomma, 2006). Once penetrated, they traverse the cortex by intracellular or intercellular growth. By three days post-inoculation, some hyphae may be able to penetrate the endodermis cells.

The inner tangential walls of epidermal cells, gum-like deposits in the cell walls of cortical cells, and the endodermis may present barriers to the successful colonization of the root by hyphae (Born, 1974). The pathogen spreads rapidly upward in the vascular tissues as result of transport of the conidia by the transpiration stream, and mycelium eventually colonizes all part of the plants as tissues turn necrotic (Emechebe, Leakey et al., 1975). One growing season is enough for the pathogen to spread throughout the above-ground parts of its host due to the transport of conidia in the transpiration stream, even in large trees. In elm (*Ulmus* spp.), conidia of *V. dahliae* need as little as eight to fifteen seconds to ascend 1.524 meters in the xylem, and they can reach an upward distribution of 6.096 meter in less than one minute by the same mechanism (Banfield, 1941). Following mycelial colonization, the conidia are produced on conidiophores or by budding of the colonizing hyphae. Some environmental factors, including low oxygen levels and a negative pressure potential, can stimulate hyphal growth inside the xylem vessels of the tree (Hiemstra & Harris, 1998).

Several resistance mechanisms in the plant can limit the colonization of *V. dahliae* inside the xylem, such as physical entrapment and vascular occlusion, and biochemical mechanisms (phytoalexins, for example) which are produced as defense responses in infected plants (Beckman, 1987). In resistant-type interactions between the plant and the pathogen, infected plants may form gel plugs and tyloses that completely block off the lumen of infected vessels above the trapping sites, so this block will immobilize the pathogen (Beckman, 1987).

The infected plant often exhibits vascular browning, a classic symptoms of vascular infection, due to activation of several secondary metabolites such as oxidized polyphenols (tannins) and phytoalexins which are exuded from paratracheal parenchyma, along with gel plugs and tyloses (Witzell & Martin, 2008). Some papers have reported that the germination and hyphal growth of *V. dahliae* in *vitro* was greatly inhibited by the tannin fraction extracted from the stained wood of *Verticillium* wilt-affected apricot trees (*Prunus armeniaca*). Some compounds such as arjunolic acid may play an important role in resistance, as this compound was found in infected stems at fungi-toxic levels (Cooper, Flood et al., 1995). In susceptible hosts, defense reactions are the same as in resistant hosts, but the pathogen seems to be successful continuing to colonize susceptible hosts (Hiemstra & Harris, 1998).

Symptoms

The general pattern of symptoms on woody hosts of Verticillium wilt is characterized by a set of classical symptoms which differ somewhat from one tree to another, depending on the host species, severity of the attack, and environmental conditions. First, foliar symptoms begin as a slight flaccidity at the tips of leaves, and extensive wilt, chlorosis, with necrosis following as the disease continues to progress. In other hosts, the flaccid stage may be absent; therefore these hosts exhibit rapid chlorosis and necrosis only (Ash, 1999).

External Symptoms

The main acute symptoms on woody hosts are dramatic, appearing suddenly as wilting, leaf scorch, defoliation, dieback, and death of a portion of or the entire plant (Sinclair & Lyon, 2005). In addition, chronic symptoms appear as sparse foliage, stunted leaves and twigs, slow growth, leaf scorch, abnormally heavy seed crops, and dieback (Ash, 2001). Both chronic and acute symptoms may occur on the same host. In olive (*Olea* spp.), sudden wilting of branches without loss of leaves is frequently reported (Hernández, Davila et al., 1998). On most species of plants affected by the disease, the roots show no external lesions or other symptoms, and the root system has a normal size and development (Rudolph, 1929). Other kinds of external symptoms include elongated dead areas of bark on the infected branches and trunks of the maple and tulip trees (Ash, 1999).

Internal Symptoms

The most common internal symptom is discoloration of vascular tissues (primarily the xylem) on most hosts, often before external symptoms are noticed. In the sapwood, the discoloration consists of dark, elongate, necrotic zones which usually are referred to as vascular streaking (Dwinell, 1967). In tree species, the discoloration of the xylem is variable with some trees showing obvious discoloration, and other trees not, including green ash and olive (Ash, 1999; Hiemstra & Harris, 1998). The appearance of the streaking can vary depending

on host: it may appear as bright olive-green in the Norway maple, brown or black in black locust (*Robinia pseudoacacia*), brown in American elm (*Ulmus Americana*), yellowish-brown in smoke tree and cherry, and purple or bluish-brown in northern catalpa (*Catalpa speciosa*) (Ash, 1999; Pirone, 1978). For diagnosis of the disease or determination of the extent of fungal invasion within the xylem of tree, vascular discoloration is not a reliable indicator due to the ability to isolate the pathogen from sapwood which exhibits no discoloration (Ash, 1999). Further, certain other fungi and abiotic stresses can cause vascular discoloration as well (Pernezny, Roberts et al., 2003). Therefore, positive diagnosis can be difficult; for example, green ash and olive often show no vascular discoloration, and isolation of the pathogen from these hosts normally is not successful (Ash, 1999; Sinclair & Lyon, 2005). Epstein and Beede isolated *V. dahliae* from one entirely asymptomatic piece of wood, and they isolated *V. dahliae* from some asymptomatic portions of wood, which suggested that colonization by *V. dahliae* does not appear to be entirely limited to the symptomatic regions of the xylem of infected trees (Epstein, Beede et al., 2004). Further, Rowe and Powelson in 2002 described that vascular discoloration symptoms on potato (*Solanum tuberosum*) were not diagnostic because the same symptoms can result from stress factors unrelated to the disease (Rowe & Powelson, 2002)

II. Management of Verticillium Wilt

Physical Methods

Several recommendations have been suggested to control or reduce the potential infection by *V. dahliae* in woody hosts, such as: avoiding intercropping with plants susceptible to *V. dahliae*; controlling weeds; avoiding or minimizing cultivation practices that damage roots; avoiding using vehicles or machinery that have been used in *V. dahliae* infested fields; using drip irrigation instead of furrow or flooding irrigation to reduce dissemination of the pathogen; removing infected shoots and branches as soon as possible, preferably before defoliation occurs; and destroying diseased material to prevent newly produced microsclerotia from being incorporated into the soil (Termorshuizen, Davis et al., 1998).

Use of heat treatment to control *V. dahliae* is a very common technique in the greenhouse. Different temperatures have been reported as thermal lethal points for *Verticillium* species (Pullman, DeVay et al., 1981). These lethal temperatures are dependent upon many factors, including the duration of the temperature during the day. Some research has shown that *V. dahliae* can survive up to 80°C in soil (Pegg & Brady, 2002). In a specially constructed apparatus, agricultural soil samples which contained survival structures of various fungal crop pathogens including *V. dahliae, Sclerotinia sclerotiorum, Sclerotium cepivorum, Pythium ultimum,* the potato cyst nematodes *Globodera rostochiensis*

and *G. pallida*, and the weeds *Chenopodium album* and *Agropyron repens* [*Elymus repens*],were treated with an aerated steam at different temperatures between 40-80ºC; the authors found that all weeds, fungi and fungal-like pathogens, and nematodes were killed by steaming at 50ºC or 60ºC for 3 min followed by an 8-min resting period in the steamed soil and immediate removal from the soil thereafter. Using the above protocol, the number of microsclerotia of *V. dahliae* in soil was significantly reduced by steaming at 45°C (Pegg & Brady, 2002). In water, the lethal dose of heat for hyphae and conidia was a minimum of 5 min at 47°C, whereas 50°C for 10 min was required to kill microsclerotia in soil (Pegg & Brady, 2002; Van Loenen, Turbett et al., 2003). Direct flaming of plants residues infested with *V. dahliae* was used to reduce the inoculum load in soil, and it reduced the number of viable propagules from two million to 5,000 per gram of soil (Beckman, 1987). Elimination of spores and microsclerotia of *V. dahliae* from tubers of *Liatris spicata* (gray flower, blazing star) was successful by using post-harvest, hot-water treatment (HWT) at 49°C for 40 min (Gilad & Borochov, 1993). In cotton waste, *V. dahliae* was eliminated by composting the plant residue when the temperature reached 50°C for four days in compost or 68°C for 12 days in soil (Pegg & Brady, 2002). In the lab, PDA cultures of *V. dahliae* isolated from olive were killed by treatments at 40°C or greater for 15 minutes or longer (Pegg & Brady, 2002). Similarly, *V. dahliae* could not been isolated from infected olive trees after the trees were subjected to 45°C or greater for 15 min, or longer (Al-Ahmad, 1993). Moreover, in several

crops, *V. dahliae* could be eliminated from diseased plant debris by burning. Levels of soil inoculum and plant infection could be reduced by stubble-burning of the potato haulm debris after three years without fumigation, which increased the yield of potato by 16%, but the combination of both burning and fumigation reduced the soilborne inoculum in some samples to zero (Pegg & Brady, 2002).

Clear polyethylene covers has been used to raise the soil temperature to eliminate soilborne inoculum not only of *Verticillium* species but also other pathogens such as nematodes as well as weed seeds (Rubin & Benjamin, 1984). In the Jordan Valley, irrigated soil was covered with a 0.03-mm polyethylene sheet during July and August to reduce inoculum of *V. dahliae* buried at various depths in covered and uncovered soil. Investigators found the temperature reached 45-48°C at 5 cm and 40-48°C at 15 cm below the surface after 2 weeks, which eliminated the pathogen to a depth of 25 cm. Furthermore, not only was *V. dahliae* killed in this treatment, but weed seeds were killed as well, and this technique enhanced potato yield by 300% (Katan, Greenberger et al., 1976). Using black plastic instead of clear plastic provided no reduction of Verticillium wilt incidence and had no effect on the yield of eggplant (*Solanum melongena*) (Moorman, 1982). In the San Joaquin Valley of California, studies have shown differences in soil temperature between the black polyethylene films and clear plastic in plantings of apple (*Malus domestica*), pecan (*Carya illinoinensis*), and grapevine (*Vitis californica*). Researchers found that soil temperature under the black plastic was increased only by 8-12°C at 15-23 cm, whereas the temperature

under clear plastic was increased by 10-18°C at the same depth; inoculum levels of *V. dahliae* under clear plastic during the winter or summer were reduced 55% to 97% at depths of 0-23 cm (Stapleton, Paplomatas et al., 1993). Some experiments have shown that the combination of solarization and fumigation is an effective method to control *V. dahliae* in soil (Pegg & Brady, 2002). Three commercial olive orchards infested with *V. dahliae* in southern Spain were used to evaluate soil solarization in four field, and in all cases, solarization experiments significantly reduced pathogen populations in the top 20 cm of soil for at least 3 years, compared with control plots (Lopez-Escudero & Blanco-Lopez, 2001).

Chemical Methods

The principal chemical control used against *Verticillium* species is fumigation, which targets inoculum in the soil or growing substrate. Other chemical approaches include spraying chemicals on the aerial parts of an infected plant or applying fungicides to soil as granular formulations or as drenches. Some experimental chemicals have been described as alternative chemical agents such as compounds that are not directly fungicidal, but which can alleviate the symptoms (Langcake, 1981). Systemic chemicals that penetrate the vascular system may also be useful. Systemic agents may have several modes of action against *V. dahliae* including counteracting pathotoxins, enhancing the resistance level of the host, and killing or suppressing the causal agent of Verticillium wilt directly within the plant (Beckman 1987).

For fumigant chemicals, many soil fumigants have been used to control *Verticillium* species by reducing inoculum levels in the soil. The most common fumigant previously used was methyl bromide (CH_3Br), which is a broad-spectrum biocide. Methyl bromide has been injected in the soil at a 15-20 cm depth, and it can permeate the soil pore space as a gas. This fumigant works effectively in moist soil because it is highly water-soluble; therefore it is important to maintain appropriate moisture content in the soil to prevent pore blockage. Fumigated soil should be covered with an impermeable polyethylene tarp for at least 48 h minimum to maintain the concentration of the methyl bromide at effective levels in the soil. By testing the efficacy of methyl bromide under a range of polyethylene sheet thicknesses from 0.0254 to 0.1524 mm, researchers found that its retention in soil was proportional to sheet thickness used in the experiment (Pegg & Brady, 2002). Methyl bromide is effective against *Verticillium* spp. in the soil, but there is a major concern with danger of toxic bromine residues accumulating in subsequent crop tissues, especially in crop rotations that include strawberry (Pegg & Brady, 2002). In recent years, methyl bromide (CH_3Br) has been phased out in under the Montreal Protocol (an international agreement ratified by more than 180 countries) because of its ability to deplete the stratospheric ozone layer, and the amount of methyl bromide produced and imported in the U.S. was reduced gradually until it was phased out completely in January 1, 2005 (U.S. EPA Methyl Bromide Phase Out Web Site at http://www.mbao.org/mbrqa.html). In addition to alternative fumigants,

alternative application methods and nonfumigant approaches have been investigated to replace methyl bromide in the management of soilborne pests (Weiland, Littke et al., 2013).

A fumigant, chloropicrin, called “tear gas”, possesses both toxic action and powerful lachrymatory action. It was tested as a soil fumigant because large stocks of this material remained after World War I (Pegg & Brady, 2002; Russell, 1920). The same guidelines apply to its use as to methyl bromide. Methylisothiocyanate (MIT) compounds which are mostly water-soluble biocides such as metam sodium (sodium N-methyldithiocarbamate) have been used against *Verticillium* species. Metam sodium was applied via sprinkler irrigation against *Verticillium* spp., but researchers found that it was effective only in arid regions (Gerstl, Mingelgrin et al., 1977; Pegg & Brady, 2002).

The role of nematode infection in the incidence or degree of symptom expression of Verticillium wilt disease has been described in a large number of papers. The association between the fungus and nematode is complex and depends upon many factors such as the species of the nematode, inoculum load, host resistance to the nematode, soil pH, soil type, and other environmental factors (Beckman, 1987; Bergeson, 1972; Powell, 1971). A variety of modes of interaction between the nematode and fungus have been discussed, including increased fungal dispersal by carrying fungus spores from one place to another, facilitating fungal penetration of host roots by creating wounds, and modifying plant tissue in such a way that it becomes a better substrate for the fungus

(Bergeson, 1972). Therefore, nematicides have been used against *Verticillium* species as soil fumigants due to the intimate relationship between the nematodes and *Verticillium* spp. (Pegg & Brady, 2002). Other nematicides have been used effectively against *V. dahliae*, including 1, 2-dibromo-3-chloropropene (Nemagon®) and 0, 0-diethyl0-[p-(methylsulfmyl) phenyl] phosphorothioate (Dasanit®) in cotton fields to eliminate soil-borne inoculum of *V. dahliae* in India (Shanmugam, Vinayagamurthy et al., 1977).

In the laboratory or in the field, a large number of experimental compounds have been investigated, with variable results. Quaternary ammonium compounds were tested *in vitro* against *V. dahliae* (Pegg & Brady, 2002). Other fungal inhibitors, especially polyamine synthesis inhibitors such as difluromethylornithine (DFMO), were used to inhibit mycelial growth at concentrations as low as 5 fM (Pegg & Brady, 2002). *In vitro*, some experiments have shown that *V. dahliae* was strongly inhibited by tannic acid (Cheo, 1982). In other experiments, ammonia and nitrous acid generated from nitrogenous amendments such as meat and bone meal were examined for their capacity to control soilborne pathogen *V. dahliae in vitro* (Tenuta & Lazarovits, 2002). The researchers found that microsclerotia of *V. dahliae* were killed at 2.5% (w/w) of meat and bone meal in an acidic loamy sand soil within two weeks due to accumulation of ammonia and nitrous acid in soil, but at lower concentrations of meat and bone the microsclerotia were killed after two weeks. On sunflower in Canada, researchers found that *V. dahliae* was inhibited by two sequiterpene

lactones, five terpenoids, and three diterpene acids in the range of 10-100 ppm (Picman, Schneider et al., 1990).

Fertilization Methods

Green manures have been used in controlling pathogen populations by plowing down the crop while still green and incorporating it into the soil. Green manures may increase total, innocuous fungal populations which may play an important role in reducing soilborne pathogens in soil simply though competition (McGuire, 2003). Several plants species have been used as green manure to suppress populations of *V. dahliae* in soil with variable results, including several legumes, vegetables, and cereal crops (Berlanger, 1999). Disease severity has been reduced by using sudangrass and other green manures (Berlanger, 1999). Sudangrass (*Sorghum vulgare* var. *Sorghum sudanense* 'Monarch') green-manure treatments added to the soil 2-3 years prior to growing potato suppressed Verticillium wilt caused by *V. dahliae* by 52% in field experiments in Idaho, compared with a plant-free control treatment (Davis, Huisman et al., 1996). The same experiment was conducted in the Columbia Basin of Washington, but they found that disease severity was actually increased by 34% over the untreated control, apparently due to environment differences between Idaho and Washington (Davis, Pavek et al., 1994; Tjamos, Rowe et al., 2000). The survival stage of *V. dahliae*, microsclerotia, were killed by adding a liquid swine manure to acidic soils, and the researchers found volatile fatty acids (VFAs) in the manure

were responsible for killing microsclerotia in soil (Tenuta, Conn et al., 2002). The use of green manures of sudangrass (*Sorghum sudanense*), wheat (*Triticum* spp.), and sweet corn (*Zea mays*) successfully suppressed nematodes, weeds, and soilborne fungal pathogens, including *V. dahliae* (McGuire, 2003). A field study evaluated the influence of using of a broccoli (*Brassica oleracea*) green manure on the microsclerotial population of *V. dahliae* and on the occurrence of Verticillium wilt on three hosts including peppermint (*Mentha* spp.), potato (*Solanum tuberosum*), and red maple. The researchers found that the population of *V. dahliae* microsclerotia in soil was decreased by 30% following the incorporation of a broccoli green manure (3.87-4.63 kg fresh weight/m^2), compared with the untreated control. Further, as result of that treatment, Verticillium wilt severity was reduced by up to 40% on potato, and red maple showed no symptoms, compared with the control. However, peppermint showed no reduction in disease severity from green-manure treatment (Berlanger, 1999). Broccoli residues were used successfully in the Salinas Valley to reduce disease incidence and severity caused by *V. dahliae* in cauliflower, compared with no broccoli residues (Koike & Subbarao, 2000). In a 3x3 factorial design, the effects of green manures of buckwheat, canola or fallow controls were tested with crop sequences of alfalfa-potato, corn-potato, and potato-potato in a 2-year field trial, the researchers found that the lowest Verticillium wilt ratings were obtained in tubers grown in soil treated with buckwheat green manures, and both highest yield and lower Verticillium wilt ratings were achieved by the crop sequence of

potato grown after either alfalfa or corn, but not after potato (Wiggins & Kinkel, 2005). In a three-year field study planted with continuously cropped Russet Burbank potato, nitrogen and phosphorus fertilization treatments suppressed Verticillium wilt of potato by 95%, and reduced the rate of increase of inoculum of *V. dahliae* in soil (Davis, Stark et al., 1994). Other reports claimed that application of nitrogen to the field can reduce disease severity, and can help to increase yield in fields infested by *V. dahliae* (El-Zik, 1985).

Biological Methods

Many biological controls of *V. dahliae* target its microsclerotia because it is the most important survival structure of the pathogen, and it plays an important role in initiating primary infection in the disease cycle. Several strategies have been proposed or tested to control or target the microsclerotia of *V. dahliae*. One possible mode of action against the microsclerotia of *V. dahliae* is to inhibit the formation of microsclerotia in infected, moribund plant tissues; however, there is no published instance of this (Tjamos, Rowe et al. 2000). Additional ways to target microsclerotia of *V. dahliae* include reducing the level of surviving microsclerotia after disintegration of infected plant tissues, preventing germination of microsclerotia on root tips, and preventing root infection by germinated microsclerotia via fungal or bacterial antagonists (Tjamos et al., 2000). In the greenhouse, soil infested with *V. dahliae* was planted with eggplant seedlings. Then, plants were inoculated with ascospores of the facultative

mycoparasite *Talaromyces flavus* as a soil drench, which resulted in a 13-56% reduction in wilt disease on eggplant plants compared with untreated control (Fahima & Henis, 1990). A report by Klinger et al (1971) showed that *Erwinia carotovora* was present in the rhizosphere of cotton that had escaped *Verticillium* infection, and they showed that *E. carotovora* was antagonistic to *V. dahliae* in the laboratory (Klingner, C. et al., 1971). *In vitro* studies have also found that *V. dahliae* was sensitive to inhibition by *Rhizobium* and 15 strains of *Agrobacterium tumefaciens*. Also, on Norway maple and silver maple seedlings, wilt symptoms were reduced by introducing into stem wounds, cells of *Bacillus subtilis* originally isolated from healthy maple stems (Pegg & Brady, 2002). Treating seed tubers of potato with strain M-4 of *Pseudomonas fluorescens* prior to planting in infested soil increased potato plant height and shoot weight and reduced density of soilborne *V. dahliae* propagules (Leben, Wadi et al., 1987). *Bacillus subtilis, Pseudomonas fluorescens,* and *Stenotrophomonas maltophilia* have been described as strong antagonists against *V. dahliae,* prompting ultrastructural and morphological changes in the pathogen (Berg, Knaape et al., 1994). For antagonists, some reports claimed fungi were more commonly antagonistic to *V. dahliae* than bacteria and actinomycetes (Pegg & Brady, 2002). Some species of fungi such as *Aspergillus ochraceus, Aspergillus terreus, Aspergillus sulphureus, Gliocladium roseum, Gliocladium virens, Myrothecium verrucaria, Myrothecium roridum, Penicillium patulum, Trichoderma harzianum,* and *Trichoderma viride* were described to be antagonistic to *V. dahliae* in *vitro* (Sezgin, 1982). Similarly,

in some experiments researchers reported that a particular strain of *V. dahliae* associated with cotton, was strongly suppressed by *Aspergillus fumigatus, Aspergillus flavus, Aspergillus lutescens, Aspergillus nidulans, Aspergillus terreus, Fusarium equieseti, Penicillium cyclopium, Penicillium notatum, Penicillium claviforme, Penicillium rubrum, Penicillium roquefortii,* and *T. viride*. However, these fungi were only moderately active against another cotton-infecting strain of *V. dahliae*. In glasshouse experiments, 34 soil isolates of fungi were tested against a *V. dahliae* strain from eggplant. Researchers found that the most effective fungi against *V. dahliae* were *Gliocladium virens, Aspergillus alutaceus, Talaromyces flavus, Paecilomyces lilacinus,* and *T. viride,* which were able to reduce the incidence of wilt disease to 0-20% compared with 90% in the control (Picman & Schneider, 1990). Similarly, *V. dahliae* was inhibited in *vitro* and *in vivo* by *Stemphylium* spp. In addition, in dual culture, Al-Rawahi and Hancock (1998) found that *Pythium oligandrum* was parasitic on *V. dahliae*, and that the reduction of microsclerotial numbers and growth of *V. dahliae* via *Pythium oligandrum* isolates was different due to the susceptibility of *V. dahliae* isolates to the parasite, which was influenced by the temperature and matric potential (Al-Rawahi & Hancock, 1998). Also, two isolates of *Bacillus spp.* (designated as K-165 and 5-127) originally isolated from the rhizosphere of infected tomatoes and eggplants were described as antagonistic bacteria against *V. dahliae* due to their ability to reduce symptoms caused by *V. dahliae* (Tjamos et al., 2000). In a three-year study, a recent report showed that increasing the

population of adult earthworms in *Verticillium*-infested field plots of eggplants reduced disease estimates of Verticillium wilt in all three years, and raised eggplant growth and yield in two out of three years (Elmer & Ferrandino, 2009).

Resistant Cultivars

Use of resistant cultivars or rootstocks has been described as the most practical and economical measure for controlling Verticillium wilt in tree species (Termorshuizen et al., 1998). Various resistance mechanisms have targeted the pathogenicity of *Verticillium* species, including fungal host cell wall-degrading enzymes; especially those that degrade polygalacturonan enzymes which give the pathogen the ability to degrade the pectin of intervessel pit membranes and penetrate at the vessel ends and between contiguous vessels (Tjamos et al., 2000). Under controlled conditions, the resistance to a non-defoliating (ND) isolate (V4) and a cotton-defoliating (D) (V117) isolate of *V. dahliae* was evaluated in 23 important olive cultivars in four experiments, and the olive cultivars were divided to two groups based on the resistance level to the two *V. dahliae* pathotypes. Cultivar group number one, including several important Spanish cultivars, was susceptible or extremely susceptible to both isolates of *V. dahliae*; however group number two exhibited some resistance based on pathotype of *V. dahliae* (Lopez-Escudero, Del Rio et al., 2004). Transgenic tomato plants (*L. esculentum*) exhibited high levels of resistance against *V. dahliae* race 2 by transferring an *ACIDIC ENDOCHITINASE GENE* (*pcht28*) isolated from *Lycopersicon chilense*

using *Agrobacterium*-mediated gene transfer (Tabaeizadeh, Agharbaoui et al., 1999). The level of tolerance to the *V. dahliae* isolate evaluated depended on exhibition of foliar disease symptoms, vascular discoloration, and vascular discoloration index, compared with nontransgenic plants. Similarly, resistant transgenic strawberry plants for *V. dahliae* were generated by inserting the same *CHITINASE GENE* (*pcht28*) using *Agrobacterium*-mediated gene transfer and a stipule regeneration method (Chalavi & Tabaeizadeh, 2003). A locus known as *Ve*, responsible for resistance against race 1 of strains of *V. dahliae* and *V. albo-atrum*, was found in tomato (*Solanum lycopersicum*) and compared to two closely linked inversely oriented resistance genes, *Ve1* and *Ve2*, that encode cell surface receptor proteins of the extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins (Fradin, Zhang et al., 2009). Further, the resistance to race 1 strain of *V. dahliae* and *V. albo-atrum* was conferred by *Ve1* (but not *Ve2*) but not against race 2 strains. In the greenhouse experiments, 16 cultivars of lettuce (*Lactuca sativa L.*) were evaluated with two races of *V. dahliae* (race 1 and race 2) to test for resistance, and the researchers found that seven cultivars were resistant to race 1. However, all cultivars were susceptible to *V. dahliae* race 2 (Hayes, Vallad et al., 2007).

III. Traditional Detection Methods for *V. dahliae*

A variety of traditional techniques have been developed for detection of *V. dahliae*; all are time consuming to apply. One way to detect the presence of *V.*

dahliae in woody hosts is to isolate and culture the pathogen from the infected woody plant tissues, which could take between 12-17 days. Moreover, using culture-based techniques, including selective media to isolate *V. dahliae*, can be challenging because *Verticillium* species grow slowly in culture, compared with other microorganisms which normally overrun the growth plates before *V. dahliae* colonies can be detected and confirmed morphologically (Ausher, 1975).

Detection of Microsclerotia from the Soil

A technique was developed to isolate microsclerotia of *V. dahliae* from soil, using two membrane filters covered with porous glass discs buried in soil for up to 5 months. Then, the germinated microsclerotia on the filter membrane were viewed microscopically (Pegg & Brady, 2002). Soilborne conidia and chlamydospores of *V. dahliae* and *V. nigrescens* have been separated successfully from microsclerotia by filtering a ×10 dilution of aqueous suspension of soil through Whatman No. 5 paper (Isaac, Fletcher et al., 1971; Pegg & Brady, 2002). The microsclerotia and conidia of *V. dahliae* in soil were trapped, using nylon monofilament fabrics with a 30-µm pore size for microsclerotia and 1-µm pore size for conidia; these were subsequently visualized by scanning electron microscopy (Lumsden, 1981). Several techniques have been described to recover the microsclerotia of *V. dahliae* from soil , using wet-sieving of soil samples followed by plating on selective media (Pegg & Brady, 2002). In 1977 Butterfield and DeVay modified the Andersen Sampler method to increase the

accuracy and sensitivity of the soil assay to detect microsclerotia of *V. dahlia* in the soil. They also found that their modified method yielded approximately 2.8 times more propagules per gram of soil than the standard wet-sieving technique (Butterfield & DeVay, 1977). Another technique used to quantify microsclerotia of *V. dahliae* from soil was called a flotation technique, and it included shaking 5 g of soil in a separatory funnel with 20 ml 1:1 (w/v) cesium chloride, which has high specific gravity, low viscosity, and low toxicity toward microsclerotia of *V. dahliae*. This procedure separated microsclerotia from the soil particles, but it was possible to recover only 55% of the microsclerotia added to the soil (Ben-Yephet & Pinkas, 1976).

Selective Media Methods

An alcohol agar medium was described as a selective medium; a 1:50 soil dilution was plated onto 15 ml of ethanol-streptomycin agar containing an additional 50 ppm penicillin in order to isolate *Verticillium* species from the soil (Pegg & Brady, 2002). Using different concentrations of glucose in pure culture was used to determine the relationship between the growth of *V. dahliae* mycelium and the production of pigmented microsclerotial material (Hall & Ly, 1972). In 1975, a selective medium was developed to isolate *V. dahliae* from senescent infected tomato tissue heavily colonized by the pathogen; this selective medium successfully suppressed the growth of saprophytic fungi, especially *Fusarium* species, using a combination of low incubation temperature (18°C) and

medium ingredients such as ethanol (0.5%), pentachloronitrobenzene (50 ppm) and antibiotics. In addition, the visualization of *V. dahliae* colonies was improved by the addition of sucrose (0.75%) and Czapek's salt (Ausher, Katan et al., 1975). In 1997, two semi-selective agar media-ethanol agar media (EA) and a modified soil extract agar media (MSEA) were used to study *V. dahliae* and *V. tricorpus,* and the two species were distinguished depending upon the coloration of media, colony shape and the shape and size of microsclerotia (Goud et al., 2003). For distinguishing *V. dahliae* from *V. albo-atrum* in *vitro*, a simple culture medium, prune-lactose-yeast agar medium, has been used (Pegg & Brady, 2002).

Staining Methods

An indirect enzyme-linked immunosorbent assay (ELISA) was used to detect the hyphae of *V. dahliae* in cotton root tissue, where the investigators used a soluble protein extracted from *V. dahliae* to prepare a specific rabbit antiserum (Gerik, Lommel et al., 1987). Then, antirabbit IgG conjugant was used to test for hyphae of *V. dahliae* by hydrolyzing the substrate (napthyl phosphate) to a product that can react with diazonium salt to outline the hyphae of *V. dahliae* in infected cotton tissue. An indirect ELISA was developed to detect isolates of *V. dahliae* from cotton, potato, and soil using polyclonal antisera prepared against purified mycelial proteins from *V. dahliae* (Sundaram, Plasencia et al., 1991). *V. dahliae* and *V. albo-atrum* were differentiated and detected in rose and chrysanthemum by selecting monoclonal antibodies (MAbs) and developing a

monoclonal double-antibody sandwich ELISA (DAS-ELISA) (Van de Koppel & Schots, 1995). A sensitive immunological assay was used to detect *V. dahliae* in infected oilseed rape plants in the greenhouse and the field as well as in plant debris (Cernusko & Wolf, 1997). An indirect competitive ELISA (IC-ELISA) has been used to detect vascular colonization of *V. dahliae* in 14 potato cultivars, and compared with a culture plate method (Plasencia & Banttari, 1997). *V. dahliae* was detected in cotton by using fluorescent staining such as fluorescein isothiocyanate (FLIC) (Pegg & Brady, 2002). In Turkey, researchers developed a double monoclonal antibody sandwich ELISA test for detecting Turkish isolates, obtained from olive plantations, as well as those from cotton and tomato fields (Yucel, Benlioglu et al., 2005).

IV. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a biochemical technology which is able to synthesize billions of copies of a specific region of DNA in a sample (Erlich, 1989). In 1983 the PCR technique was developed by Kary Mullis who was awarded the Noble Prize in Chemistry for this work in 1993 (Bartlett & Stirling, 2003). The story of PCR began with Friedrich Miescher, when he was able to isolate DNA from white blood cells in 1869 (Dahm, 2008). In 1950 James Watson and Francis Crick were able to solve the mystery of the structure of DNA when they published a paper to explain their model of DNA as two strands of complementary base-paired DNA, moving in opposite directions to form a double

helix structure (Watson & Crick, 1953). In 1983 Kary Mullis, PCR inventor, came up with this brilliant idea, considered to be the most important and influential discovery in the molecular biology revolution (Walker, 2002). The number of PCR applications has increased rapidly in different fields such as genetic fingerprinting of forensic samples, diagnosis of genetic diseases, genomic fingerprinting, mutagenesis and engineering of DNA, direct nucleotide sequencing of genomic DNA and cDNA, direct cloning from genomic DNA or cDNA, and diagnosis of pathogens including bacteria, viruses, and fungi (Kramer & Coen, 2002).

PCR is performed *in vitro* on a specific target DNA sequence (the DNA template) to generate a large number of identical copies of a specific portion of template, so that it can readily be analyzed. The PCR reaction requires two oligonucleotide primers that flank the DNA template to be amplified, dNTPs which included four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), a heat-stable DNA polymerase, and magnesium ions in the buffer. Also, the PCR reaction is a repetitive series of temperature cycles. PCR typically consists of 20-40 thermal cycles, and each cycle is normally a three-step process (Bermingham & Luettich, 2003). The first step, called denaturation, separates the double-stranded DNA into single strands by heating the mixture to between 92°C to 98°C. In the second step (annealing step), the temperature is reduced to between 37°C to 55°C (the actual temperature depends upon the sequences and length of the primers) in the presence of oligonucleotide primers which bind (=

anneal) to their complementary sequence on the DNA template (the denatured single-stranded DNA). Finally, in the elongation or extension step, the temperature is increased again to 60-72°C which is optimal for DNA polymerases that extend the primers by adding nucleotides (dNTPs) to the 3' end in manner complementary to the corresponding template strand, resulting double-stranded DNA molecules identical to the original DNA template (Bermingham & Luettich, 2003). Theoretically, in an efficient PCR, after twenty cycles, the PCR will yield about a million (2^{20}) copies of the amplicon (Erlich, 1989). The PCR products can be visualized or identified by the amplicon size using agarose gel electrophoresis, comparing with a DNA ladder of known fragment size (DeTrana, 2007).

For PCR specificity, primers should be designed flanking the target, and their sequence should be unique in the sample genome. The length of primers should be between 14-40 nucleotides with balanced G+C content near 50%; they should not produce stable secondary structures; and they should not be complementary to each other in order to avoid forming primer dimers (Bermingham & Luettich, 2003). A PCR protocol is specific when only one amplification product is produced of the target sequence. Temperature, salt concentration, and appropriate conditions in the PCR reaction, play important roles in the interaction between the primers with intended target sequence versus non-target sequence that may also be present in extracts of sample DNA (Arnheim & Erlich, 1992). Studies indicated that there was a relationship among

the PCR specificity, its efficiency and its fidelity (Cha & Thilly, 1993). If a PCR protocol can amplify more copies of the product with fewer cycles, it is considered to be highly efficient. A PCR protocol is considered to be high-fidelity to the extent that it has a low error rate during the extension step, and a high degree of accuracy in the replication of the DNA of interest (Frey & Suppmann, 1995). High specificity, efficiency (i.e. yield), and high-fidelity will generate the ideal PCR. These three parameters are affected by many factors such as the nature of the target sequence, each component of the PCR reaction, the thermocycling system, and buffer conditions. Unfortunately, each parameter has its own, undefined, ideal condition; therefore the condition that will give high efficiency (yield) may not be compatible with the condition that will give us a PCR with high specificity or fidelity; therefore it is important to plan to optimize a PCR protocol beforehand, depending on the PCR application (Cha & Thilly, 1993). For instance, in our lab, my project focuses on the detection of *V. dahliae* by PCR in woody hosts; therefore high specificity PCR is more important than high-fidelity or efficiency of PCR.

PCR has undergone many improvements to generate a technique called real-time polymerase chain reaction or quantitative polymerase chain reaction (qPCR). Researchers reported that the level of initial template concentration could be estimated based on the number of cycles required for amplification to exceed background (Rasmussen, 2001). In real-time PCR, amplification can be monitored in real-time, using specific florescent probes or a nonspecific

fluorescent DNA binding dye (Higuchi, Fockler et al., 1993; Holland, Abramson et al., 1991). In comparison to conventional PCR, real-time PCR uses the florescent signals of the labeled amplification products in the PCR as the real-time detection system (DeTrana, 2007). As a result, the need for gel electrophoresis to detect amplification products was eliminated by the combining DNA amplification and detection into a single PCR assay (Bustin, 2005). The number of cycles required to yield enough fluorescent signals to exceed background is called the cycle threshold (C_t); this indicates the degree of success in amplification of target DNA. Quantitative polymerase chain reaction (qPCR) is the use of real-time PCR to estimate initial template concentration by taking advantage of the normally linear relationship between the $\log_{10}$ of the initial concentration of target DNA and the cycle threshold (C_t) in the PCR reaction. In other words, the higher initial concentration of DNA template, the earlier amplification will be detected, and a lower cycle threshold (C_t) value indicates a higher initial concentration of target DNA (Bustin, 2005).

In real-time PCR, several fluorescence-based chemistries are used for monitoring the DNA amplification, including SYBR® Green dye 1, Taqman® probes, and molecular beacons (Walker, 2002). SYBR® Green dye 1 emits fluorescence nonspecifically, and only when it bound to double-stranded DNA; therefore an increase in SYBR® Green dye 1 fluorescence signals indicates that amplification is occurring, but the florescent signal may not be due to the target

amplicon. In contrast, with a well-designed Taqman® probe, an amplification signal typically means that the target amplicon is being amplified.

For PCR applications in plant pathology, real-time PCR has emerged as a powerful tool for plant certification, plant quarantine, pathogen identification, disease management, and crop production (Martin, James et al., 2000). This detection method provides several advantages over traditional diagnosis methods because it does not require isolation and culturing the fungus and it is sensitive, rapid, and reliable (DeTrana, 2007; Zheng, Chen et al., 2005).

V. DNA-Based Detection of *V. dahliae*

PCR is widely used as a powerful tool for detection, identification, and detection of harmful plant pathogens, including bacteria, viruses, and fungi (Lopez, Bertolini et al., 2003). PCR assays have been developed for detection and quantification of fungi, including *V. dahliae*, in a variety of hosts. For example, two primers (NMS1/NMS2) were designed based on the mitochondrial small rRNA gene region of *V. dahliae*, and used to amplify a specific fragment of the *V. dahliae* genome, which is about 140 bp (Li, Rouse et al., 1994). A quantitative PCR (qPCR) assay was used with primers specific for *V. dahliae* to evaluate the amount of colonizing biomass of *V. dahliae* in resistant and tolerant cultivars of potato (Dan, Ali-Khan et al., 2001). PCR assays have also been used to detect the presence of the pathogen in symptomless plants (Dan et al., 2001). A

conventional PCR assay was developed to screen red chicory plants (*Cichorium intybus*) infected with *V. dahliae* before using those plants for seed production, because some infected plant are symptomless (Mascarello, Favaron et al., 2001). In 2003, a duplex, nested- PCR assay was developed to identify the defoliating (D) and non-defoliating (ND) *V. dahliae* pathotypes in olive trees even before the plants exhibited symptoms (Mercado-Blanco, Rodriguez-Jurado et al., 2003). A PCR-based assay depends on a single set of primers, a nested PCR-based assays that has more than one primer set, and standard culture-plating procedure were compared for detection of *V. dahliae* in olive trees, especially in asymptomatic olive trees at different tree heights; the researchers reported that PCR based a single set of primers exhibited higher sensitivity than culture-plating procedure especially in symptomatic trees and at most heights (Karajeh & Masoud, 2006). Moreover, the nested PCR-based assay exhibited higher sensitivity than a single PCR assay even in asymptomatic tree and at all heights. A nested PCR procedure with specific primers designed from the internal transcribed spacer (ITS) regions of nuclear ribosomal RNA (rRNA) genes has been used to detect the presence of *V. dahliae* in symptomatic and asymptomatic nursery-propagated olive plants, and compared with standard plating for detection of *V. dahliae* (Karajeh, 2006). In 2007, a multiplex real-time quantitative PCR with specific primers (VertBt-F/VertBt-R) designed from the sequence of the βate-tubulin gene, was used to detect and quantify colonization of *V. dahliae* in potato plants exhibiting early dying disease, caused by *V. dahliae* (Atallah, Bae et al., 2007). In Northwest

Spain, a real-time PCR procedure was used to detect *V. dahliae* in pepper samples even when those samples were asymptomatic (Gayoso, de la Ilarduya et al., 2007). In Japan, a PCR assay was applied as a detection and identification tool to distinguish different pathotypes and races of *V. dahliae* in tomato and sweet pepper (Usami, Ishigaki et al., 2007). A multiplex-nested-PCR assay-based 'molecular tool box' was used to detect vegetative compatibility groups (VCGs) of *V. dahliae* infecting artichoke plants, even in symptomless plants, in order to study the genetic and pathogenic diversity of *V. dahliae* population that infected this host (Collado-Romero, Berbegal et al., 2009). Another PCR assay was developed to study the genetic variability and race structure of *V. dahliae* isolates that infected different hosts in central and costal California (Maruthachalam et al., 2010). A new method for quantifying microsclerotia formation by *Verticillium* species in soil was developed based on real-time PCR assays with primers that were designed to the ribosomal DNA internal transcribed spacer for *V. tricorpus* and the *BATE-TUBULIN GENE* for *V. dahliae* and *V. longisporum*. Researchers found that the real-time PCR based assay was specific, and faster than the wet-sieving method, and that it could differentiate the three species of *Verticillium* in soil (Debode, Van Poucke et al., 2011). A PCR assay was optimized to detect and quantify *V. dahliae* in spinach germplasm and in 15 commercial spinach seed lots produced in the United States or Europe to help in making the decision as to whether to apply seed treatment, depending on the incidence of *V. dahliae* infection in the seeds (Duressa, Rauscher et al., 2012). Quantitative real-time

PCR assay using primers and a Taqman probe specific for *V. dahliae* was developed to detect and quantify the inoculum density of *V. dahliae* in the soil in association with a wide range of hosts, including strawberry (Bilodeau & Koike, 2012). A set of primers was designed from the *TRYPSIN PROTEASE GENE* of *V. dahliae* to generate a qPCR protocol for detection and quantification of *V. dahliae* in potato stem (Pasche, Mallik et al., 2013). A quantitative nested real-time polymerase chain reaction (QNRT-PCR) has been used to detect the presence of *V. dahliae* DNA in soil in smoke tree nurseries or in infected plant tissues; the assay was able to detect the soil inoculum densities as low as 1 microsclerotium/g of soil, so it was suggested as tool in disease control measures for Verticillium wilt by permitting assessment of the risk of *V. dahliae* infection of smoke trees before planting (Wang, Wang et al., 2013).

CHAPTER TWO: Evaluation of PCR-Based Methods for Rapid, Accurate Delectation and Monitoring of *Verticillium dahliae* in Woody Hosts by Real-Time Polymerase Chain Reaction

Introduction

Verticillium wilt of woody hosts, caused by the soil-borne pathogen *Verticillium dahliae* Klebahn, is an economically important disease not only in North America but worldwide (Bhat & Subbarao, 1999). *V. dahliae* has a broad host range, including economically important vegetables, field crops, forest trees, and woody and herbaceous ornamentals (Berg, Fritze et al., 2001; Klosterman et al., 2009; Pegg & Brady, 2002). The fungus can survive in the soil for up to 14 years as microsclerotia, which serve as the principle pathogen propagule to disperse among fields (Fradin & Thomma, 2006). Microsclerotia are stimulated to germinate by root exudates (Ben, Toueni et al., 2013; Hu, Bai et al., 2013; Pasche, Thompson et al., 2013). Germinated microsclerotia produce hyphae to penetrate roots at the root tip, the zone of elongation, and physical injuries, including wounds caused by nematodes. Once penetrated, the mycelium crosses the endodermis to access to vascular (xylem) tissues (Fradin & Thomma, 2006). During hyphal colonization, conidia are formed in vessel elements, and are transported in the transpiration stream, ultimately clogging vessel elements, resulting in plants showing symptoms.

The practical importance of early detection of *V. dahliae* in woody plants becomes more valuable due to the challenges of controlling this disease. Verticillium wilt of woody hosts has been considered as one of the most difficult plant diseases to control due to several factors, including long-term survival of the pathogen in soil as microsclerotia, its broad host range, the inability of fungicides to achieve suppressive concentrations during pathogen colonization of the xylem, the inability to implement crop rotation, and the long life of its woody hosts (Termorshuizen et al., 1998). Further, resistant varieties of woody host species are not available for Verticillium wilt. Finally, no highly effective therapy exists for trees infected with *V. dahliae,* so the infected tree must be replaced (Ash, 1999).

For the purposes of research or diagnosis, hosts infected by *V. dahliae* exhibit specific symptoms that are used to presumptively diagnose the disease, including external symptoms (wilt, chlorosis, necrosis, and dieback) and internal symptoms (vascular discoloration). However, foliar disease symptoms (wilt, chlorosis, necrosis, and dieback) are not a reliable indicator of infection by *V. dahliae*, as the symptoms it induces may also result from a variety of biotic and abiotic stresses (Plasencia & Banttari, 1997). For instance, cold winters may cause symptoms on twigs which are similar to Verticillium wilt symptoms. Also, vascular discoloration may be used as an indicator of *V. dahliae* infection, but certain fungi and abiotic stresses can cause the same symptoms as well (Almeida, 2009). Conversely, the fungus can be isolated from sapwood which exhibits no

vascular discoloration (Ash, 1999). In addition, some woody hosts exhibit weak to no vascular discoloration when infected. Therefore, vascular discoloration should not be relied on for diagnosis either (Ash, 1993; Pernezny et al., 2003). Another technique used to detect *V. dahliae* in woody hosts is based on isolation of the fungus in pure culture, a process which can take several days. Further, using selective media to isolate *V. dahliae* can be challenging because *Verticillium* species grow slowly, compared with other microorganisms which may overrun plates before *V. dahliae* colonies can be detected (Ausher et al., 1975). Furthermore, dormancy of microsclerotia or inhibition of growth of *V. dahliae* by other microorganisms on semi-selective media may cause variable and false-negative results (Li et al., 1994). While isolation in culture can be a useful tool for detecting this fungus from some hosts, it is not commonly successful from certain hosts, such as green ash and olive (Ash, 1999). Given this, a rapid and accurate real-time PCR-based assay for detection of *V. dahliae* in woody hosts would be of great value.

Polymerase chain reaction (PCR) has emerged as a powerful pathogen detection method in crop sciences, including certification programs, plant quarantine, pathogen identification and diagnosis, disease management, and crop production (Martin et al., 2000). Using specific primers to amplify a particular DNA target from a species of interest is the cornerstone of a PCR-based diagnostic assay (Gayoso et al., 2007). PCR offers several advantages over traditional detection methods because it does not require isolation and culturing of

the fungus from infected plant tissues. Furthermore, PCR typically is sensitive, rapid, and reliable (Gayoso et al., 2007; Zheng et al., 2005). Real-time PCR was developed for even faster detection and quantification of plant pathogens including bacteria, viruses, and fungi (McCartney, Foster et al., 2003). Compared to conventional PCR, real-time PCR techniques offer advantages including reduced contamination risks, eliminating the need for ethidium bromide staining, and removing the time and cost of gel electrophoresis (Mumford, Walsh et al., 2000). In recent years, several PCR-based assays have been used to identify and quantify *V. dahliae* on different hosts (Atallah et al., 2007; Bilodeau & Koike, 2012; Dan et al., 2001; Debode et al., 2011; Duressa et al., 2012; Gayoso et al., 2007; Pasche, Thompson, et al., 2013; Wang et al., 2013). However, to our knowledge, existing PCR primer/probe sets have not been compared, and none of those assays were tested against woody plants.

The objectives of this research were: (i) to evaluate published pairs of species-specific primers for detecting *V. dahliae*, in order to develop a rapid and accurate tool for detection and screening for the presence of *V. dahliae* in various woody hosts and (ii); to evaluate methods of disrupting woody plants tissues for DNA extraction.

Materials and Methods

Fungal Isolates

In all experiments, four isolates of *V. dahliae* and one isolate of *V. albo-atrum* were used to develop a PCR assay specific for *V. dahliae*, and two other organisms included *Phytophthora capsici* and *Pyricularia oryzae* were used as negative controls. All isolates and their origin are listed in Table 2-1. All fungal isolates were maintained as spores at -80°C in potato dextrose broth with 50% (v/v) glycerol, and they were grown on potato dextrose agar (PDA) for three days by pipetting approximately 25 µl of the glycerol/media spore suspension onto PDA plates. The identification of isolates was confirmed by morphological analysis and by direct sequencing of both ITS and VMSP regions (described below). Morphological analysis to the level of the genus was conducted by growing on Czapek-Dox agar (CDA) in the dark for 10 days at the room temperature (Dhingra & Sinclair, 1985). The presence of conidia and conidiophores typical of the genus was confirmed by microscopic observation. Verification to the level of species was based on production of microsclerotia (*V. dahliae* isolates #64114, #884, and #891) or melanized hyphae (*V. albo-atrum* isolate # V.10, IPP 032) on PDA. Microscopic examinations were made after 12 days of incubation. All *Verticillium* spp. isolates with globose, oval to elongate and abundant black microsclerotia without melanized mycelium were verified as

V. dahliae (Goud et al., 2003). The isolate producing melanized hyphae was considered to be *V. albo-atrum* (Goud et al., 2003).

Fungal DNA Extraction

Fungal isolates were grown at room temperature for 10 days in Petri dishes containing PDA. Plugs were aseptically cut from the actively growing margins of the fungus colonies, and transferred into flasks containing 20-25 ml of sterile potato dextrose broth (PDB). After 7 days of incubation in the dark at room temperature, mycelium was harvested by filtering through sterile filter paper and rinsing with sterile distilled water. Approximately 100 mg of mycelium (fresh weight) was added to 2.0-ml screw cap microcentrifuge tubes containing two 4-6 mm sterile glass beads. Then, mycelium was frozen in liquid nitrogen and ground into a fine powder using a bead-beater at 2500 rpm for 30 s (Mini-Bead Beater, Biospec Products 3110BX). For DNA extraction, the pulverized mycelium was suspended in 1 ml of extraction buffer [2% (w/v) cetyltrimethyl ammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP-40), 100 mM Tris-HCl pH 8, 1.4 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8], with 2 µl 2-mercaptoethanol, and 10 µl of 100 mg/ml Qiagen RNase A solution added immediately before mixing]. The solution was mixed by vortex and incubated at 65°C for 15 min with gentle mixing 2-3 times during the incubation, followed by microcentrifugation at 10,400 rpm for 10 min. After centrifugation, 650 µl of the supernatant was transferred to a 1.5 ml microcentrifuge tube and mixed with equal volume of 24:1 (v/v) of

chloroform/isoamylalcohol]. The solution was centrifuged at 12,500 rpm for 10 min. Then, 500 µl of the supernatant was transferred to a 1.5 ml microcentrifuge tube contained 350 µl of isopropanol, gently mixed, and centrifuged at 12,500 rpm for 10 min. Pellets were rinsed with ice-cold 70% (v/v) ethanol, centrifuged at 12,500 rpm for 5 min and air-dried. The pellets were dissolved in 100 µl of surplus Buffer AE from the Qiagen DNeasy kit (10 mM Tris, and 0.5 Mm EDTA, pH 9.0). The resuspended DNA was stored in a -20ºC freezer (Li, Mock et al., 2008; Mascarello et al., 2001).

Molecular Verification of *V. dahliae* Isolates

In order to verify the identity of isolates of *V. dahliae*, extracts of genomic DNA were amplified via PCR using primers ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White, Bruns et al., 1990) in a GeneAmp® PCR system 9700 thermocycler set at max ramp rate. Moreover, specific primers for *V. dahliae* VSMP1 (5'-CATAAAAGACTG CCTACGCCG-3') and VSMP2 (5'-AAGGGTACTCAAACGGTCAG-3') were used to amplify all isolates of *V. dahliae*, from which all amplicons were submitted for direct sequencing (Mascarello et al., 2001) to the University of Kentucky Advance Genetics Technology Center (UK-AGTC). PCR experiments were conducted in 25-µl reaction volumes. Each reaction mixture contained: 0.5 µM of each primer, 0.5 U Titanium Taq DNA polymerase, 12.5 µl of FailSafe™ PCR Premix Selection Kit H (60U Enzyme mix and 12 premixes cat no: FS99060, Epicentre Technologies), approximately 20 ng of template DNA, and

sterile, molecular-grade water (sufficient to bring the final volume of the reaction to 25 µl). Cycling conditions for amplification included an initial denaturation at 95°C for 120 sec followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. The reaction products were resolved by electrophoresis in 2% (w/v) agarose and visualized by UV-fluorescent staining with ethidium bromide. The size of the fragments was determined by comparison with an O'Gene Ruler 100 bp (Genenco) DNA ladder. For direct sequencing, PCR amplicons were resolved in 2% (w/v) Nusieve GTG Agarose with 1X TAE buffer, removed using a clean, sharp scalpel under UV light and stored in a 1.5 ml microcentrifuge tube at 4°C. DNA was obtained from gel slices using the QIAquik® Gel Extraction Kit (Qiagen) as follows: gel slices were weighed in colorless tubes. Three volumes of QG buffer were added to one volume of gel slice (100 mg gel ≈100 µl). Gels slices were incubated at 50°C for 10 min and the tubes were vortexed 2-3 times during the incubation until the gel slices were completely dissolved. Then, one volume of isopropanol was added to each tube and mixed carefully. The samples were applied to the QIAprep® spin columns (Qiagen) and centrifuged for one min at 13,000 rpm to bind the DNA. Flow-through was discarded and the QIAprep® spin columns were placed back into the same tubes. QG buffer (500 µl) was added to each sample, which was then centrifuged for one minute at 13,000 rpm. Flow-through was discarded and the QIAprep® spin columns were placed back into the same tubes. PE buffer (750 µl) was added to the QIAquik columns, centrifuged for one min at 13,000 rpm to

wash the the QIAprep® spin columns. The flow-through was discarded. Columns were incubated for 2-5 min after addition of buffer PE, then, placed into clean 1.5 ml microcentrifuge tubes. EB buffer (50 µl) (10 mM Tris HCl., pH 8.5) was added to the center of the QIAprep® spin column membrane, centrifuging for one min at 13,000 rpm to elute the DNA solution. DNA samples were stored at -20°C until submission for direct sequencing, which was done at the UK-AGTC.

Evaluation of Published Specific Primer Sets for *V. dahliae*

Several published primers sets specific for *V. dahliae* (Table 2-2) were evaluated against known *V. dahliae* isolates originally obtained from infected woody hosts (Table 2-1). The specificity of all primer sets was evaluated by real-time PCR in 25-µl reaction volumes in a Cepheid Smart Cycler® II thermocycler. All primer sets were evaluated using thermocycling conditions specified in the papers cited in Table 2-3. Four of six primer sets (VertBt-F/VertBt-R, VDS1/VDS2, VMSP/VMSP2, and DB19/DB22) were tested using SYBR® Green as a reporter dye. The reaction mixture contained approximately 20 ng of DNA template, 1X PCR buffer, 0.5 U of Titanium Taq, 0.2 mM of dNTPs (Promega), 0.2 µM of each primer, 2.5 µl of 5X SYBR® Green, 5 µl Smart Cycler Additive Reagent (SCAR), and sterile molecular-grade water to 25 µl. As a negative control, sterile molecular-grade water substituted for DNA template in one reaction. In all assays, which were performed at least twice, reaction products were resolved in 1.5 % (w/v) agarose gel, and the fragments were visualized under UV-fluorescent after staining with ethidium bromide. Fragments sizes

were estimated using a 100 bp DNA O'Gene Ruler ladder. Also, for SYBR® Green-based assays, melt curve analysis (ramp rate 0.2 C/s) was used to evaluate for potential primers dimers, nonspecific amplification, and amplification of target amplicons from *V. dahliae*, *V. albo-atrum*, *P. capsici*, and *P. oryzae*. Three primer sets were tested with their associated Taqman® probe (each labeled with FAM and BHQ-1): DB19 plus DB22 with probe espdf01, Vd-F929-947 plus Vd-R1076-1094 with *V. dahliae* probe, and VTP1-2F plus VTP1-2R, with VTP1-2P probe, as described by the authors (Table 2.3).

Alignment of Target Sequences of Published Primers

DNA sequences delineated by primer sets evaluated in this study were aligned in order to evaluate the degree of sequence identity in the primer-binding sites of isolates of *V. dahliae* and other related *Verticillium* species. A ClustalW alignment of all primers evaluated in this study was generated with sequences of related *Verticillium* spp., when such sequences were available. Sequences were downloaded from the National Center for Biotechnology Information (NCBI) database. All sequences were aligned using the Bioedit sequence alignment editor program, and forward and reverse primers were indicated on the alignment.

In order to generate the alignment for VMSP1 and VMSP2, which were designed to amplify a region in the mitochondrial small rRNA gene, nine strains of *V. dahliae,* two strains of *V. albo-atrum,* one strain *V. nubilum,* one strain of *V. fungicola*, one strain of *V. nigrescens*, one strain of *V. theobromae*, and one strain

of *V. tricorpus* were used this alignment. For the alignment of primers VertB-F and VertB-R, designed from the β-tubulin gene of *V. dahliae*, 12 strains of *V. dahliae*, two strains of *V. albo-atrum*, one strain of *V. longisporum*, one strain of *V. tricorpus*, and one strain of *V. nigrescens* were downloaded and aligned. Primers Vd-F929-947 and Vd-R1076-1094, and their corresponding probe were designed from intragenic spacer 65 (IGS) sequences; therefore corresponding sequences were downloaded and aligned for 15 strains of *V. dahliae*, one strain of *Verticillium albo-atrum*, one strain of *V. fungicola*, one strain of *V. longisporum*, one strain of *V. nubilum*, one strain of *V. nigrescens*, and one strain of *V. tricorpus*. An alignment for primers DB19 and DB22, and their corresponding probe (espdfo1), was generated using sequences of six strains of *V. dahliae*; corresponding sequences for related *Verticillium* species were not found in the NCBI database. Primers VTP1-2F and VTP1-2R, and their corresponding probe (VTP1-2P), target the *TRYPSIN PROTEASE GENE* (*VTP1*) of *V. dahliae*; sequences from one strain of *V. dahliae* and one strain of *V. albo-atrum* were downloaded and aligned.

PCR Sensitivity

The sensitivity of two primer sets (VertBt-F/VertBt-R andVDS1/VDS) to the presence of host DNA (maple) was tested using varying concentrations of DNA of *V. dahliae* isolate #876 at (per 25-µl reaction) 20 ng, 2 ng, 0.2 ng, 0.02

ng, 2 pg, 0.2 pg, 0.02 pg, and 2 fg. Results were evaluated using cycle threshold (C_t) values and melt curve analysis.

Disruptions of Woody Plants Tissue and Its Impact on DNA Extraction

Elimination of PCR inhibition and good quality nucleic acid are critical to apply PCR technique for diagnosis (MacKenzie, McLean et al., 1997). Nucleic acids were obtained from samples of a maple tree and an ash tree located outside the Plant Science Building on the campus of the University of Kentucky, Lexington. Twigs segments 20 to 30 cm in length were removed from the trees and surface-sterilized with undiluted household bleach. Bark and phloem were removed under aseptic conditions to avoid contamination with other extraneous DNA (Zhang, Uyemoto et al., 1998). Xylem tissue was mechanically disrupted in a 2 ×3 factorial design, as follows. Primary xylem tissue disruption was performed in two ways and resulted in small wood shavings:

- Twigs were shaved using a mechanical pencil sharpener (APSCO standard sharpener) (Fig. 2.1). To minimize cross contamination between samples, the shaving head was thoroughly washed in a detergent solution and rinsed in water (Green, Thompson et al., 1999).
- Xylem was drilled using a 0.3 cm drill bit (Fig. 2.2). The bit was washed with ethanol (70% v/v) to minimize cross-contamination among samples.

After primary xylem disruption, three levels of secondary xylem tissue disruption (pulverization) were evaluated:

- Approx. 100 mg of wood shavings were added to a mesh plastic bag (item number ACC00930/0100; AgDia Inc.) (Fig. 2.3), and mixed with 3 ml of CTAB buffer, 2 µl of mercaptoethanol, and 10 µl of 100 mg/ml RNase. This mixture was mashed for 2-3 min using a drill press fitted with a ball-bearing tool (Tissue Homogenizer; item number ACC 00900; AgDia Inc.) designed to compress the mesh bags (Fig. 2.4). One ml of homogenate was added to a 1.5 microcentrifuge tube, and DNA was extracted using the CTAB method described above.
- Wood shavings were grounded into a fine powder using a Mini-BeadBeater-1, (Biospec Products 3110BX) using the following steps (Fig. 2.5):
    1. One-hundred mg of wood shavings and two stainless steel beads (5 mm, Qiagen) were added to a sterile 2-ml bead-beater tube (catalog number 72.693; Sarstedt).
    2. The tissue was frozen by placing it in liquid nitrogen for 2 min.
    3. Tubes were transferred to a Mini-BeadBeater-1, and ground for 30 s at 4,200 rpm shaking-speed.

4. DNA was extracted using the CTAB method, as described above.

- No secondary tissue disruption.

Following DNA extraction using the CTAB method described above, the concentration of DNA obtained was quantified using an Invitrogen Qubit® fluorometer (Life Technologies), as per manufacturer's instructions (probes.invitrogen.com/qubit).

Evaluation for PCR Inhibition

The potential presence of PCR inhibitors is high because woody plant tissues often contain high concentrations of phenolic compounds and polysaccharides inhibitory to enzymes used in PCR (Bessetti, 2007; Demeke & Adams, 1992; Osman & Rowhani, 2006). During PCR, false negatives can result from the presence of PCR inhibitors co-extracted with DNA obtained from plant tissue. Tests for this were conducted using two primers sets (VertBt-F/VertB-R and VDS1/VDS) and extracts of woody plants. DNA extracts were obtained using the CTAB method described above, from PCR-negative samples collected from six species: sugar maple (*Acer saccharum),* ash (*Fraxinus species*), oak (*Quercus pubescens*), redbud (*Cercis canadensis*), crabapple (*Malus* spp.), and barberry (*Hydrastis canadensis*). To test for inhibition, PCR reactions containing 3 µl of DNA extract from woody plants plus 20 ng of genomic DNA of *V. dahliae* isolate #876 were compared to reactions containing 20 ng of *V. dahliae* alone. The nuclei of *V. dahliae* are haploid and mycelial cells are generally

monokaryotic; therefore, adding 20 ng of genomic DNA was eqivalent to 5.44 X $10^{\wedge 5}$ molecules, which would be equal to 5.44 X $10^{\wedge 5}$ cells of *V. dahliae* per PCR reaction. Then, PCR reactions were compared using C_t values and melt curve analysis.

Application of Selected Real-time PCR Assays to Field-Grown Trees

Samples were collected from two sugar maple trees exhibiting symptoms of wilt, chlorosis, epinasty, and stunting but no vascular discoloration; these were obtained from Spy Coast Farm in Lexington, Fayette County, Kentucky (Fig. 2.6). Two asymptomatic trees were sampled from the same location (Fig. 2.7). Also, two five-year-old smoke trees (*Cotinus coggygria*) with foliar symptoms typical of Verticillium wilt and with vascular discoloration were sampled from Frankfort, Kentucky (Fig. 2-8). Eight twigs (40 cm) were arbitrarily collected from different positions of each tree, and these were surface-sterilized in 0.04% (v/v) NaOCl for 5 min. Then, sample tissue was shaved using a drill bit, followed by pulverization of the wood shavings by using the bead-beater; then, DNA extraction was conducted as described above. Extracted DNA from each twig was tested using primer sets VertBt-F/VertB-R andVDS1/VDS. Moreover, all samples obtained from trees were tested for the presence of PCR inhibition by comparing C_t values obtained using 20 ng of DNA extract from known *V. dahliae* isolate #876 to that obtained in tubes containing DNA of both *V. dahliae* #876 plus sample DNA, in order to test for false negative reactions with these samples. Shavings from several twigs were aseptically plated onto 1.5 % (w/v) water-ager medium, and

incubated in darkness at room temperature for 1-2 weeks. Colonies of *V. dahliae* were identified based on colony morphology.

In addition to these assays, both primer sets were tested against a variety of woody samples suspected to be affected by Verticillium wilt and submitted to the University of Kentucky Plant Diagnostic Laboratory. Moreover, all samples were tested for the presence of PCR inhibitors as described above.

Confirmation of Target Amplicon Generated Using Primer Sets VertBt-F/VertBt-R and VDS1/VDS1

In order to verify the accuracy of the two most promising primers sets in this study (VertBt-F/VertBt-R andVDS1/VDS), amplicons that were obtained from several PCR-positive woody samples were evaluated. For confirmation of the accuracy of VDS primers, two positive samples (#781 and # 2188) were amplified using VDS primer set. Then, amplicons generated using VDS primer set were confirmed by direct DNA sequencing after gel purification, as described above. For VertBt primers, the direct sequencing method was unsuccessful due to amplicon shortness; therefore confirmation of amplicon identity was done using restriction enzyme analysis. NEBcutter, version 1.0, (http://tools.neb.com/NEBcutter) was used to identify enzyme restriction sites and predict fragment sizes for the expected amplicon. This analysis was conducted with a sequence of the *V. dahliae* β-tubulin gene (Vd_215: AACAACAGTC CGATGGATAATTCTCAGCAGCATTTGCTCATGGTTTTCTTTCTTTG

CAGGCCTCTGGCAACAAGTACGTTCCCCGTGCCGTCCTCGTCGATCTC GAGCCCG GTAC), downloaded from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The restriction enzyme HaeIII, which recognizes the sequence, 5'...GGCC... 3', was chosen to cleave the 115 bp-amplicon into fragments of 58 bp and 57 bp in length. Restriction reactions were conducted as recommended by the manufacturer (New England BioLab® Inc.), using 100 ng of amplicon per reaction. The restriction enzyme products were visualized in 3% (w/v) agarose gel by UV- fluorescence after staining with ethidium bromide, and compared with a 100 bp DNA O'Gene LR ladder.

Results

Identification of Isolates of *V. dahliae*

The identity of *V. dahliae* isolates used in this study (Table 2.1) was confirmed using cultural characteristics (conidial and conidiophore morphology, microsclerotial morphology) (Fig. 2.9). The morphological characters of *V. albo-atrum* were confirmed by the production of brown, pigmented hyphae typical of *V. albo-atrum* on potato dextrose agar (PDA) after 12 days of growth at room temperature in the dark (Fig. 2.10). For *V. dahliae* isolate #64114, BLAST searches of the direct sequences of ITS region obtained using primers ITS1 and ITS4 provided matches of between 96-100% to *V. dahliae* accessions. However, ITS direct sequencing provided no significant similarity with any sequence in the

NCBI database for the remaining isolates of *V. dahliae* (#891, 884, and 876) due to the poor quality of sequences obtained. For direct sequences obtained using VMSP primers, a BLAST search provided at or near 100% identity of 60-70% of the total amplicon length to *V. dahliae* accessions for all isolates of *V. dahliae*.

Evaluation of Published Primer Sets Specific for *V. dahliae*

All primer sets were evaluated against a set of isolates of *Verticillium* spp., and these primer sets provided mixed results. The SYBR® Green-based assay using the VertBt primer set amplified all isolates of *V. dahliae* with cycle threshold C_t values between 16 to 22; amplification was also observed in the negative controls and with the *V. albo-atrum* isolate (Fig. 2.11). However, the melt derivative curves revealed the species-specificity of this assay by showing the melt temperatures for amplicons of all *V. dahliae* isolates, which were approx. 86°C, whereas all the negative controls plus the *V. albo-atrum* isolate exhibited melt temperatures of approx. 79°C, typical of primer-dimers and short non-specific amplification products (Fig. 2.12). Moreover, using gel electrophoresis, the primer pair VertBt amplified a unique DNA fragment of the expected size of approx. 115 bp from the DNA extracts of all *V. dahliae* isolates, and no amplicon was observed with the isolates of *V. albo-atrum*, *P. capsici*, or *P. oryzae* (Fig. 2.13). Results of using this primer set were consistent over two experiments, and the entire amplification time was about 50 min, which is the shortest among all primer sets tested.

The SYBR® Green-based assay with VDS primers amplified all isolates of *V. dahliae* with C_t value approx. 23 to 28; *V. albo-atrum* and negative controls were amplified with C_t values approx. in the range of 32 to 33 (Fig. 2.14). However, the specificity of VDS primers was exhibited by the melt temperatures which were between 87-88°C for all isolates of *V. dahliae* and between 77-76°C for *V. albo-atrum* isolate plus the negative controls (Fig. 2.15). VDS primers produced the expected DNA fragment of approx. 541 bp from the DNA extracts of all *V. dahliae* isolates over two experiments (Fig. 2-16). DNA extracts of *V. albo-atrum*, *P. capsici*, and *P. oryzae* (*data not shown*) yielded no amplicon.

The primer pair VSMP1 and VSMP2 amplified a DNA fragment of approximately 140 bp, the expected size of the amplicon from this primer set, but one *V. dahliae* isolate (#64114) consistently produced a double band (Fig. 2.17). However, these primers did not amplify the isolates of *V. albo-atrum*, *P. capsici*, or *P. oryzae* (*data not shown*). Primers DB19 and DB22 consistently produced an amplicon of the expected size in some isolates of *V. dahliae* but not in others (Fig. 2-18). The remaining primers sets tested (Vd-F929-947/ Vd-R1076-re, and primers VTP1-2F/VTP1-2R, each set with its corresponding Taqman® probe) failed to amplify the expected DNA fragment from the DNA extracts of all *V. dahliae* isolates tested (Fig. 2.19 and 2.20).

Alignment of Target Sequences of Published Primers

In the alignment of rDNA sequences, the target of primers VSMP1 and VSMP2, the binding site of the forward primer (VSMP2) was identical in eight of nine *V. dahliae* accessions, with the Vd_K12 accession showing a polymorphism at the 3' end (Fig. 2.21). This polymorphism also differentiated this primer from all accessions of other *Verticillium* spp. included in the alignment. The reverse primer (VMSP1) was identical to the target sequence in all nine accessions of *V. dahliae,* but the primer also exhibited 100% identity to the two accessions of *V. albo-atrum* (Vaa_1776 and Vaa_CBS7) and the accession of *V. nubilum* (Vn_IMI_27) across its full length. However, the reverse primer exhibited mismatches in both ends of the primer against *V. fungicola* (Vf_IMI_18), *V. nigrescens* (Vni_71799), *V. theobromae* (Vth_IMI_2), and *V. tricorpus* (Vt_188) (Fig. 2-21).

In the alignment of the β-tubulin gene, the target of VertBt primers, the forward primer (VertBt-F) was conserved in all 12 strains of *V. dahliae* at both 3' prime end and 5' end (Fig. 2.22). However, internally, the forward primer exhibited a Single-nucleotide addition in three accessions of *V. dahliae* (Vd_250, Vd_538, and Vd_226). The forward primer exhibited a single nucleotide difference with two strains of *V. albo-atrum* (Vaa_PD620 and Vaa_TASVa) at the 5' end. The 3' end of VertBt-F has shown several mismatches the strains of *V. albo-atrum*. This primer exhibited 100% identity against one strain of *V. longisporum* (Vl_PD356in) across its full length. The 5' end of primer exhibited

even more mismatches against other stains of *Verticillium* species, including the strain of *V. tricorpus* (Vt_TASVt2) and the strain of *V. nigrescens* (Vni_V51). Further, it exhibited even more mismatches against the strain of *V. tricorpus* (Vt_TASVt2) and the strain of *V. nigrescens* (Vni_V51). Thus, with the exception of *V. longisporum*, the alignment suggested that this primer is unique to *V. dahliae*. The binding site for the reverse primer (VertBt-R) was identical in all but one of the 12 accessions of *V. dahliae*, with the exception (Vd_219) exhibiting a polymorphism in two nucleotides. The reverse primer exhibited 100% identity with strains of other of *Verticillium* spp., including two strains of *V. albo-atrum*, the strain of *V. longisporum*, and the strain of *V. tricorpus*, indicating that this primer is not unique to *V. dahliae*. The binding site of the reverse primer differed substantially from the sequence of *V. nigrescens*.

In the alignment of the IGS target of primers Vd-F929-947 and Vd-R1076-1094 with their corresponding probe, all three oligos exhibited 100% identity to all 15 strains of *V. dahliae*, and they exhibited mismatches against other *Verticillium* species included in the alignment, including one strain of *V. albo-atrum* (Vaa-VICVa), one strain of *V. fungicola* (Vf-NBAII), one strain of *V. longisporum* (Vl-Bob69), one strain of *V. nubilum* (Vn-CABI), one strain of *V. nigrescens* (Vnl-CABI), and one strain of *V. tricorpus* (Vt-So959) (Fig. 2.23). In an alignment of sequences serving as the target of primers DB19 and DB22 and their corresponding probe (espdfo1) the forward, reverse primer were identical in all six strains of *V. dahliae* evaluated; however, the binding side of the probe

(espdfol) exhibited a substantial deletion in three out six strains of *V. dahliae* (Fig. 2-24). Sequences for this target of other *Verticillium* species were not available in Genbank, so an *in silico* evaluation of oligo specificity was not possible. An alignment of the *TRYPSIN PROTEASE GENE* (*VTP1*) exhibited 100% identity between VTP1 oligos and one strain each of *V. dahliae* and *V. albo-atrum* suggesting a lack of species specificity (Fig. 2.25). No additional sequences for this primer set were found in Genbank, precluding additional *in silico* analysis.

Real-time PCR Sensitivity

Using the VertBt-F and VertBt-R primers, the relationship between the C_t value and template concentration was nearly linear over the range of concentrations of 20 ng to 20 pg per reaction (Fig. 2-26), with a calculated PCR efficiency of E= 0.99 over this range. The minimum quantity of *V. dahliae* DNA that could be detected with this primer set was 2 pg per reaction. The nuclei of *V. dahliae* are haploid, and except at hyphal tips, its cells are monokaryotic; therefore this PCR protocol using VertBt primer set allows the detection of approximately 54.4 *V. dahliae* genome equivalents (which would be the DNA expected from about 54.4 *V. dahliae* cells) per 25 µl-reaction. Using the VDS primers, C_t values were nearly linear over a range of template concentrations of 20 ng to 2 pg. In other words, the PCR protocol using VDS primer set detected about 5.44 *V. dahliae* genome equivalents (which would be the DNA expected from approximately 5.44 cells of *V. dahliae*) per 25 µl-reaction (Fig. 2.27). For VDS1 and VDS2 primers the detection limit was 0.2 pg of *V. dahliae* DNA per

reaction. With either primer set, the presence of host DNA (maple) did not influence the real-time PCR efficiency, as indicated by the similarity of C_t values over a wide range of template concentrations (Figs. 2-26 and 2-27).

Disruption of Woody Tissue and Its Impact on DNA Extraction

Two notable ANOVA interactions were significant ($P<0.05$): Host*PTD (Host= ash or maple, PTD= primary tissue disruption) and Host*PTD*STD (STD= secondary tissue disruption) (Table 2.4). Consequently, means for different levels of host and tissue-disruption method were analyzed separately. Use of either a mechanical pencil sharpener or drill bit to disrupt the xylem of twigs was successful in producing fine shavings as a primary tissues distribution step (Fig. 2.28). Following this step, pulverized wood was obtained from the shavings using a bead beater or drill press with mesh bags, followed by DNA extraction using the CTAB method. Using different secondary tissue disruption methods provided different concentrations ($P=0.0126$) and the interaction among the host species, secondary tissue disruption, and primary tissue disruption was significant as well ($P=0.0061$). Primary tissue disruption using a drill bit followed by processing with a bead beater provided the highest DNA concentration with oven-dried twigs from ash trees, especially when compared with yield using the pencil sharpener (Fig. 2-29). In oven-dried maple twigs, the drill bit followed by the bead beater exhibited the highest numerical mean of DNA concentration compared with other treatments, but it also exhibited high variability as reflected in the high standard error values (Fig. 2-30). In fresh ash

twigs, numerically the highest concentration of DNA was provided by the pencil sharpener followed by drill press with mesh bags, although variability was also high in that treatment (Fig. 2.31). In contrast, in fresh maple twigs, the drill bit followed by the drill press with mesh bags provided the highest DNA concentration (Fig. 2-32). Using the primary tissue disruption only (by either the mechanical pencil sharpener or drill bit), with no secondary tissue disruption, was consistently associated with low DNA yield, likely due to the lack of adequate mechanical disruption.

Evaluation for PCR Inhibition in DNA Extracts from Woody Tissues

In this study, two real-time PCR-based assays (based on primer sets VertBt-F/VertBt-R and VDS1/VDS2) were developed to detect *V. dahliae* in woody hosts. The assay with VertBt-F/VertBt-R primers was evaluated for sensitivity to potential PCR inhibitors co-purified with DNA from woody hosts, because tissues from woody plants contain high concentration of phenolic compounds and polysaccharides which can be inhibitory to DNA polymerase enzymes used in PCR (Demeke & Adams, 1992; Osman & Rowhani, 2006). Amplification of the *V. dahliae* β-tubulin gene target using VertBt-F/VertBt-R primers was generally not influenced by the presence of DNA extracts from a variety of woody plants, as reflected in C_t values similar to that of the positive control (Fig. 2-33). One woody species (crabapple tree) exhibited a detection threshold C_t value slightly higher than the positive control, but an increase in the C_t value of approximately 0.6 cycles compared to the control is not considered to

be reflective of substantial reaction inhibition. Real-time PCR using primers VDS1/VDS2 was similarly unaffected by the same DNA extracts obtained from woody species, with the exception of the extract from redbud (Fig. 2.34). However, that observed increase in C_t value was also modest and unlikely to impede detection in infected trees. These results suggest that amplification using the VertBt and VDS primers may often be unaffected by the PCR inhibitors in DNA extracts obtained from woody plants.

Application of Selected Real-time PCR Assays to Field-Grown Trees

The detection ability of real-time PCR-based assays using VertBt and VDS primer sets was evaluated with trees exhibiting different patterns of disease symptoms. Using the VertBt primer set, seven of eight twigs sampled from asymptomatic tree number one (AST1) exhibited a positive reaction, as did all eight twigs sampled from asymptomatic tree number two (AST2) (Table 2.5). All twigs sampled from maples exhibiting wilt symptoms but no vascular discoloration (WST1, WST2) also were positive, using VertBt primers. In addition, all eight twigs sampled from tree VST1 (smoke tree exhibiting wilt symptoms plus vascular discoloration) were PCR-positive using the VertBt primer set, while seven of eight twigs were PCR-positive in the second smoke tree. In addition, all the samples exhibited no PCR inhibition using the VertBt primer set, as evidenced by similar C_t values obtained using *V. dahliae* #876 in the presence or the absence of sample DNA extract (*data not shown*). In contrast, the real-time PCR-based assay with the VDS1/VDS2 primer set produced

negative results using the same DNA extracts (Table 2.5); as with VertBt primers, there was no evidence of PCR inhibition caused by DNA extracts from these woody samples in PCR reactions containing DNA of *V. dahliae* #876 (*data not shown*). In each sample where culturing was performed, colonies typical of *V. dahliae* were obtained (Table 2.5), verifying infection by *V. dahliae*.

Both primer sets were also tested with samples of a variety of woody hosts exhibiting varied symptoms typical of Verticillium wilt and submitted by University of Kentucky Extension agents (Table 2-5). Sample #1197 obtained from a smoke tree with classic symptoms of Verticillium wilt exhibited a positive result with VertBt primer set, but it was negative with VDS primers. In that case, complete PCR inhibition of the VDS primers was detected in the reaction of *V. dahliae* #884 spiked with sample DNA extract. Negative results were observed with both primer sets with samples #1615, #P-750, and #1869 sampled from a variety of woody plants exhibiting both wilt and vascular discoloration symptoms. Also, the negative results of these samples were supported by culture plating. In addition, sample #Vincelli was negative using both primer sets, and the culture plating provided no evidence of the presence the pathogen in this sample. In that case, the tree exhibited foliar wilt, rapidly progressing truck cankers, and associated discoloration of the cambium, consistent with a canker disease and not Verticillium wilt, thus negative PCR tests were consistent with field observations. Maple sample #P-781exhibited wilt symptoms but not vascular discoloration symptoms and gave a positive result with the VertBt primer set and a negative

result with the VDS primer set. Furthermore, *V. dahliae* was isolated successfully from the maple sample #P-781 using the culture plating method, corroborating the PCR results obtained with the VertBt primers. Redbud sample #1570 exhibited both classic foliar wilt and vascular discoloration symptoms, and gave a positive PCR result using both primer sets. However, isolation the pathogen from this sample was failed using the culture plating. Sugar maple sample #2188 exhibited classic wilt but not vascular discoloration yet tested positive with both primer sets and *V. dahliae* was isolated successfully using the culture plating method.

For confirmation of the amplicons observed, the amplicon obtained using the VDS1 and VDS2 primers from two PCR-positive woody host samples was confirmed via direct sequencing. The sequences generated from positive samples exhibited about 100% identity against accessions of *V. dahliae*, based on a NCBI BLAST search. The amplicon generated using the VertBt primer set was too small to direct-sequence, so restriction digestion was used to verify amplicon identity. NEBcutter, version 1.0 (http://tools.neb.com/NEBcutter), identified a restriction map of the amplicon of *V. dahliae* strain Vd_215 (Fig. 2.34). The restriction enzyme HaeIII (recognition sequence 5'...GGCC... 3') was selected to cleave the 115 bp-amplicon into two fragments of 58 bp and 57 bp. In all amplicons of the six samples tested, digestion produced fragments of the expected size obtained using the VertBt primers (Fig. 2.35).

Discussion

This study presents a detection tool for *V. dahliae* in woody hosts using real-time PCR based on the primer set VertBt-F and VertBt-R , generated from the single-copy β-tubulin gene of *V. dahliae* (Atallah et al., 2007). Also, another real-time PCR-based assay for detection *V. dahliae* in woody hosts was evaluated using the primer set VDS1 and VDFS2, designed from a specific probe sequence of *V. dahliae* (Mercado-Blanco et al., 2003). However, the VDS primer set failed to amplify from several asymptomatic or symptomatic infected woody hosts. Moreover, this primer set was sensitive to PCR inhibition with a sample from a field-grown smoke tree, which may sometimes cause false negative reactions. VSMP primers successfully amplified a single band of the expected size from several isolates of *V. dahliae*. However, they consistently amplified double bands from isolate 64114 of *V. dahliae*, which could be due to several reasons. One is that, while this study used the annealing temperature indicated in the original description (54°C), it may have been lower than optimal, which would increase the chance for primer binding to non-specific targets. Alternatively, it is possible that primer concentration was excessive, which may increase primer binding to non-specific targets as well. Given that these primers amplify repetitive gene sequences, there is the possibility that fragments of different lengths exist in the intervening sequences between the primer binding sites, which would result in bands of different sizes. The DB primer set amplified some but not all isolates of *V. dahliae* tested, which suggests this primer set is not reliable as a diagnostic tool

for Verticillium wilt in woody hosts. Since the remaining primers sets failed to amplify DNA extracts obtained from isolates of *V. dahliae*, they clearly are not suitable for diagnosis, at least under the test conditions of this study.

Several studies have used PCR-based methods for diagnosis of several tree-infecting fungal species, and such assays should have a high level of sensitivity and specificity in order to be a successful method for pathogen detection (Broders, 2010; Cho, Kang et al., 2011; Hamelin, Bourassa et al., 2000; Piskur, Ogris et al., 2007; Ramsfield, Dobbie et al., 2008). The real-time PCR-based method presented here, using the VertBt primer set, detected as little as 2 pg of genomic DNA of *V. dahliae*, both in the presence or the absence of host DNA. In addition to demonstrating a high level of sensitivity, this provides additional evidence that the VertBt primers are generally insensitive to PCR inhibitors of woody hosts, or that the technique for extracting DNA from woody hosts results in minimal amounts of inhibitory substances. Further, this set of primers was able to detect the presence of *V. dahliae* in woody samples even before the host exhibited symptoms. The real-time PCR-based method with primer set VDS1/VDFS2 detects as little as 0.2 pg of *V. dahliae* DNA genomic DNA (the equivalent of 54 cells of *V. dahliae*) and was similarly not inhibited by the addition of DNA extracts from most samples from woody species tested in this work. However, this set of primers was not able to detect the presence of *V. dahliae* in asymptomatic woody hosts, and failed to amplify samples that were positive for the presence of *V. dahliae* both by PCR using the VertBt primers and

by isolation. Moreover, the PCR-based assay with the VDS primer set was sensitive to inhibition in one sample, which suggests an increased risk of false negatives using this primer set, as compared to the VertBt primer set.

This study showed that the time needed to diagnosis *V. dahliae* in woody hosts can be reduced from between 12-17 days (required for culture-based methods) to one day, which allows time for both DNA extraction and real-time PCR using primer set VertBt-F/VertBt-R. Similarly, only one day is needed for DNA extraction time plus the real-time PCR using primer set VDS1/VDFS2, although there are other concerns previously mentioned above with these primers.

Several techniques have been used to detect *V. dahliae* in a variety of hosts. These includes culturing on a selective medium, a technique which presents certain difficulties, including being time-consuming, the limited number of samples that can be processed, and labor requirements (Plasencia & Banttari, 1997). Further, selective media lack specificity due to the slow growth of *V. dahliae*, which can be overrun by growth other microorganisms such as fungi and bacteria (Karajeh & Masoud, 2006; Plasencia & Banttari, 1997). Because of the lack of a rapid, sensitive, and specific method to detect *V. dahliae* in woody hosts, a rapid and reliable diagnostic tool for detection of *V. dahliae* in woody hosts will be useful, which may help control the movement and use of infected plant materials.

The detection of *V. dahliae* can be an important tool for Verticillium wilt management in woody hosts due to the spread of the pathogen via infected transplants used to establish new nurseries or orchards. Many factors make the control of *V. dahliae* in woody hosts difficult, including its wide host range, the ability of the pathogen to survive for years via microsclerotia in soil, the inability of chemicals fungicides to contact the pathogen in the xylem during the parasite stage, and woody hosts' long life.

In the present study, a new two-step procedure was developed to disrupt the hard xylem of woody plant tissues for DNA extraction. In primary tissue disruption, a drill bit was effective in creating small wood shavings from small twigs and branches, as a first step in tissue processing. Using the drill bit for primary tissue disruption followed by the bead beater for secondary tissue disruption yielded high DNA concentrations from oven-dried twigs of both ash and maple. In contrast, in fresh tissues, a pencil sharpener for primary tissue disruption followed by the drill press with mesh bags yielded the highest DNA concentration from ash twigs. However, this treatment exhibited high variability via showing high standard error values. With fresh maple twigs, the highest DNA concentration was obtained using a drill bit for primary tissues disruption followed by the drill press with mesh bags. In other words, both experimental factors host species and condition (dried vs. fresh) influenced the DNA yield from samples of woody plants. Further, woody tissues often have high concentrations of phenolic compounds and polysaccharides which may be inhibitory to enzymes

used in PCR. However, the drill bit followed by bead-beating presented here seems effective in minimizing or eliminating PCR inhibitors. Furthermore, both real-time PCR assays, especially the one based on VertBt primer set, were able to detect DNA of *V. dahliae* extracted from infected woody hosts, which suggests that the extracted DNA contained insignificant amounts of compounds that could inhibit the PCR reaction. In the inhibition test with varieties of woody, we spiked the woody samples with 20 ng of *V. dahliae*, which would be equivalent to 5.44 X $10^{\wedge 5}$ target molecules per PCR reaction. The high concentration of *V. dahliae* genome equivalents used in these tests could potentially overcome the influence of the PCR inhibitors in woody samples in the PCR reaction. Thus, one cannot rule out the possibility that higher levels of inhibition might occur if substantially lower concentrations of genomic DNA of *V. dahliae* were used in the presence of the same amount of host extract.

In conclusion, a powerful, rapid, and reliable molecular tool based on published primers was developed for detection of *V. dahliae* in woody hosts. A real-time PCR-based assay using primer set VertBt-F/VertBt-R exhibited greater accuracy and sensitivity than real-time PCR-based methods using five other published primer sets, and was much faster than traditional microbiological isolation procedures. This molecular tool has many applications which could be useful in plant disease diagnosis and management, especially as the most practical and economical control method of *V. dahliae* in woody hosts is avoiding planting infected transplants in newly established nurseries or landscapes (Karajeh &

Masoud, 2006). The real-time PCR-based assay using primer set VertBt-F/VertBt-R could potentially be used in screening for the presence of *V. dahliae* in nurseries or in mother trees, which could be valuable in maintaining sanitation during the establishment of new plantings. It was notable that this pair of primers was able to detect the presence of *V. dahliae* in infected asymptomatic woody samples, suggesting that this method would be able to detect the pathogen at an early stage of infection.

For future research on this topic, evaluation of the VertBt primer set using more isolates of *V. dahliae* would be valuable in order to represent potentially different clades of *V. dahliae* and different geographical areas and isolates of different host origin. It may be beneficial to evaluate these real-time PCR-based assays with a wide variety of isolates of *V. dahliae,* in order to evaluate the primer sets against the widest possible range of genetic variability in the target genome regions among *V. dahliae* isolates that infect woody hosts.

Table 2.1. Species, host of origin, year of isolation, location, and source of isolates of *V. dahliae*, *V. albo-atrum*, *Phytophthora capsici*, and *Pyricularia oryzae* used to evaluate the specificity of published primer sets to detect *V. dahliae* in woody hosts.

Species	Isolate Designation	Host of Origin	Year of Isolation	Location	Source
V. dahliae	876	Maple (*Acer sp.)*	2006	Fitchburg, Dane Co., WI.	K. Subbarao
V. dahliae	884	Ash (*Fraxinus sp*.)	2006	Nashoteh, Waukesha Co.,WI	K. Subbarao
V. dahliae	891	Maple (*Acer sp.*)	2006	Waukesha Co, WI	K. Subbarao
V. dahliae	64114	Maple (*Acer sp*.)	2003	Illinois	ATCC[1]
V. albo-atrum	V.10, IPP	Irish potato (*Solanum sp.)*	unknown	Wisconsin	A.Tiedemann
Phytophthora capsici	01-2011-G	Pumpkin (*Cucurbita sp*.)	2001	Kentucky	B. Amsden
Pyricularia oryzae	KY-96	Perennial ryegrass (*Lolium sp.)*	1996	Lexington, KY	P. Vincelli

(1) American Type Culture Collection

Table 2.2. Primer name, sequences, molecular target, fragment length and citation of all specific primers and probe for *V. dahliae* evaluated in this study.

Primers name	Primer Sequence(5'-3')	Target	Fragment Length (bp)	Citation
VMSP1 VMSP2	CATAAAAGACTGCCTACGCCG AAGGGTACTCAAACGGTCAG	rDNA gene	140	(Mascarello, 2001)
VertBt-F VertBt-R	AACAACAGTCCGATGGATAATTC GTACCGGGCTC GAGATCG	β-tubulin gene	115	(Atallah, 2007)
VDS1 VDS2	CACATTCAGTTCAGGAGACGGA CCGAAATACTCCAGTAGAAGG	DNA probe sequence	520	(Gayoso, 2007)
DB19 DB22 Espdf01	CGGTGACATAATACTGAGA GACGATGCGGATTGAACGAA [FAM(2)]TGAGACTCGGCTGCCACAC[BHQ_1(3)]	SCAR sequence (1)	540	(Mercado, 2003)
Vd-F929-947 Vd-R1076-10 *V. dahliae* probe	CGTTTCCCGTTACTCTTCT GGATTTCGGCCCAGAAACT [FAM]CACCGCAAGCAGACTCTTGAAAGCCA [BHQ_1]	Intragenic spacer (IGS)	121	(Bilodeau, 2012)
VTP1-2F VTP1-2R VTP1-2P	CTCGATCGTCGTCAACC TGGTGGTGAGAGTGTTG [FAM]TACGACAACGACTTCGCCATC [BHQ_1]	Trypsin protease (VTP1) gene	155	(Pasche, 2013)

(1) A sequence characterized amplified region (SCAR) from *V. dahliae*.

(2) Indicates a fluorophore covalently attached to the 5'-end of the oligonucleotide probe to emit the fluorescence signals.

(3) Indicates a quencher covalently attached to the 3'-end of the oligonucleotide to inhibit any fluorescence signals.

Table 2.3. Thermocycling conditions for primer/probe sets evaluated for specificity to *V. dahliae* isolates obtained from infected woody hosts.

Primers/probes used	Sequences (5'-3')	Thermocycling conditions	Citations
VMSP1 VMSP2	CATAAAAGACTGCCTACGCCG AAGGGTACTCAA ACGGTCAG	94°C for 3 min: 35 cycles at 94°C for 60 s, 54°C for 60 s, and 72°C for 2 min.	(Mascarello, 2001)
VertBt-F VertBt-R	AACAACAGTCCGATGGATAATTC GTACCGGGCTCGAGATCG	3 min at 95°C: 40 cycles of 95°C for 10 s and 63°C for 35 s.	(Atallah, 2007)
VDS1 VDS2	CACATTCAGTTCAGGAGACGGA CCGAAATACTCCAGTAGAAGG	95°C for 2 min: [40 cycles at 95°C for 30 s, 62°C for 30 s,72°C for 1 min], and 72°C for 5 min.	(Gayoso, 2007)
DB19 DB22 Espdf01	CGGTGACATAATACTGAGA GACGATGCGGATTGAACGAA [FAM]TGAGACTCGGCTGCCACAC [BHQ_1]	94°C for 4 min: [35 cycles of 1 min at 62°C, 30 s at 72°C, 1 min at 94°C], and 6 min at 72°C.	(Mercado,2003)
Vd-F929-947 Vd-R1076-1094 *V. dahliae* probe	CGTTTCCCGTTACTCTTCT GGATTTCGGCCCAGAAACT [FAM]CACCGCAAGCAGACTCTTGA AAGCCA [BHQ_1]	95°C for 3 min: 40 cycles at 95°C for 1 min, 60°C for 45 s, and 72°C for 1 min 30 s.	(Bilodeau, 2012)
VTP1-2F VTP1-2R VTP1-2P	CTCGATCGTCGTCAACC TGGTGGTGAGAGTGTTG [HEX]TACGACAACGACTTCGCCATC [BHQ_1]	2 min at 95°C: 40 cycles of 30 s at 95°C, 60 s at 58°C, and 30 s at 72°C.	(Pasche, 2013)

Table 2.4. Analysis of variance for DNA yields following disruption of woody tissue.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Host [(1)]	1	0.25399837	0.25399837	0.38	0.6022
Rep (Host)	2	1.35109008	0.67554504	2.19	0.1623
Ptd [(2)]	1	0.69326004	0.69326004	2.25	0.1645
Std [(3)]	2	4.30528075	2.15264038	6.99	0.0126
Ptd*std	2	0.28344008	0.14172004	0.46	0.6440
Host*ptd	1	2.58267204	2.58267204	8.38	0.0160
Host*std	2	0.12714525	0.06357262	0.21	0.8169
Host*ptd*std	2	5.46993958	2.73496979	8.88	0.0061

(1) Indicates host (maple vs. ash).

(2) Indicates method of primary tissue disruption (drill bit vs. pencil sharpener).

(3) Indicates method of secondary tissue disruption (drill press plus mesh bags vs. bead beater vs. no secondary tissue disruption).

Table 2.5. Comparison of real-time PCR-based assay with VertB-F/VertB-R primers and VDS1/VDS2 primers, and culture plating for detection of *V. dahliae* in trees exhibiting differing symptom types, including asymptomatic sugar maples (*Acer saccharum*) (AST), sugar maple exhibiting wilt symptoms (WST), and a smoke tree (*Cotinus coggygria*) exhibiting wilt plus vascular discoloration (VST).

		Real-time PCR Result		
Tree Symbol	**Tree Status**	**VertBt Primers**	**VDS Primers**	**Culture Plating**
AST1[1]	Asymptomatic	7/8 [2]	0/8	2/2 (12 days)[3]
AST2	Asymptomatic	8/8	0/8	NT[4]
WST1	Wilt symptoms	8/8	0/8	2/2 (12 days)
WST2	Wilt symptoms	8/8	0/8	NT
VST1	Vascular symptoms	8/8	0/8	2/2 (12 days)
VST2	Vascular symptoms	7/8	0/8	NT

[1] AST, WST, and VST indicate asymptomatic tree, tree exhibiting wilt but no vascular symptoms, and tree exhibiting wilt plus vascular symptoms, respectively. Number indicates the tree number sampled within that symptom category.

[2] Number of positive twigs/total number of twigs sampled from the same tree.

[3] "2/2" indicates two twigs positive/total numbers of twigs used to verify of *V. dahliae* by culturing and morphological examination.

[4] "NT" indicates culturing not attempted on twigs from this tree.

Table 2.6. PCR results of samples of woody hosts collected in Kentucky and submitted for diagnosis due to suspicion of *V. dahliae* infection.

Number of Samples	Host	Location	Wilt Symptoms	Vascular Discoloration	Real-time PCR Results VertBt Primers	VDS Primers	Culture Plating
#1197	Smoke tree	Nelson Co	+ [1]	+ [2]	+ [3]	-	NT[4]
#1615	Catalpa	Bath Co	+	+	-	-	-
#2232	Smoke tree	Frankfort	+	+	+	-	+
#P-750	Barberry	McCracken Co	+	+	-	-	-
#P-781	Maple	Christian Co	+	-	+	-	+
#2188	Sugar maple	Fayette Co	+	-	+	+	+
#1869	Norway maple	Oldham Co	+	+	-	-	-
#1570	Redbud	Fayette Co	+	+	+	+	-
#Vincelli	Redbud	Fayette Co	+	+	-	-	-

[1] "+" indicates twigs sampled from a tree exhibiting wilt symptoms, "-"are twigs sampled from tree exhibited no wilt symptoms.

[2] "+" indicates twigs sampled from tree exhibiting vascular discoloration, "-" are twigs sampled from tree exhibited no vascular discoloration.

[3] "+" indicates positive result with real-time PCR, "-" negative result with real-time PCR.

[4] "NT" indicates culturing not attempted on twigs from this sample, "+" indicates positive result using culture plating method, "-" negative result using culture plating method.

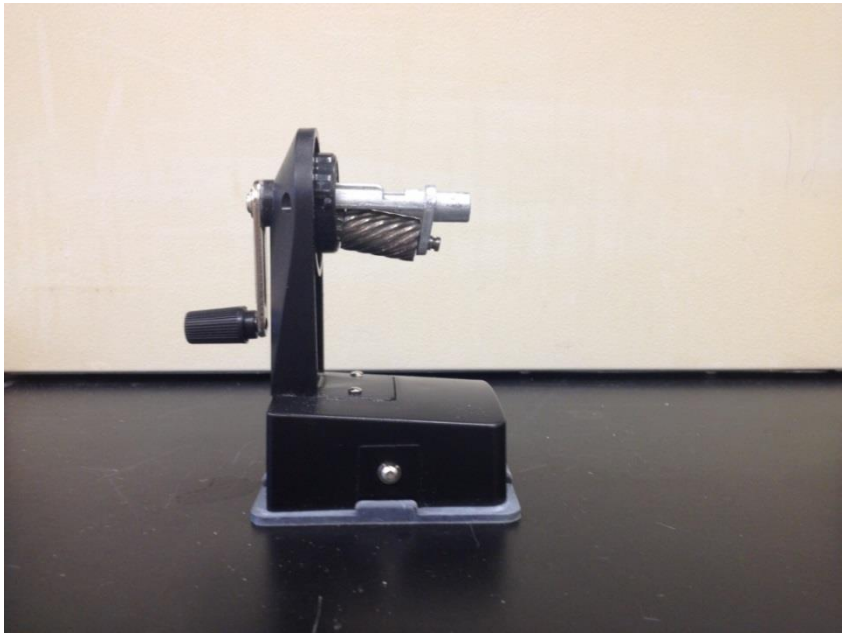


Figure 2.1. A mechanical pencil sharpener (APSCO standard sharpener) used to grind twigs into fine shavings.

Figure 2.2. Drill (Ryobi) plus 0.3-cm bit used to grind twigs into fine shavings.

Figure 2.3. The sample mesh bags (item number ACC00930/0100; AgDia Inc.) used to mash wood shavings with CTAB buffer.

Figure 2.4. Mesh bags used to process woody shavings in the CATB buffer under pressure from a Tissue Homogenizer (item number ACC 00900; AgDia Inc.).

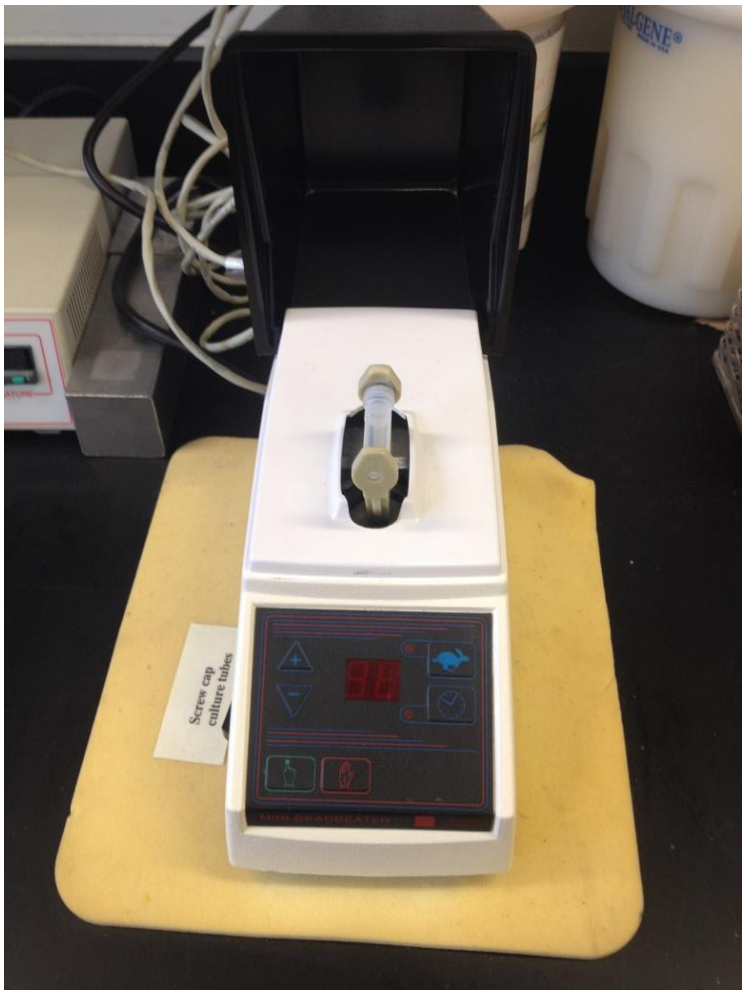
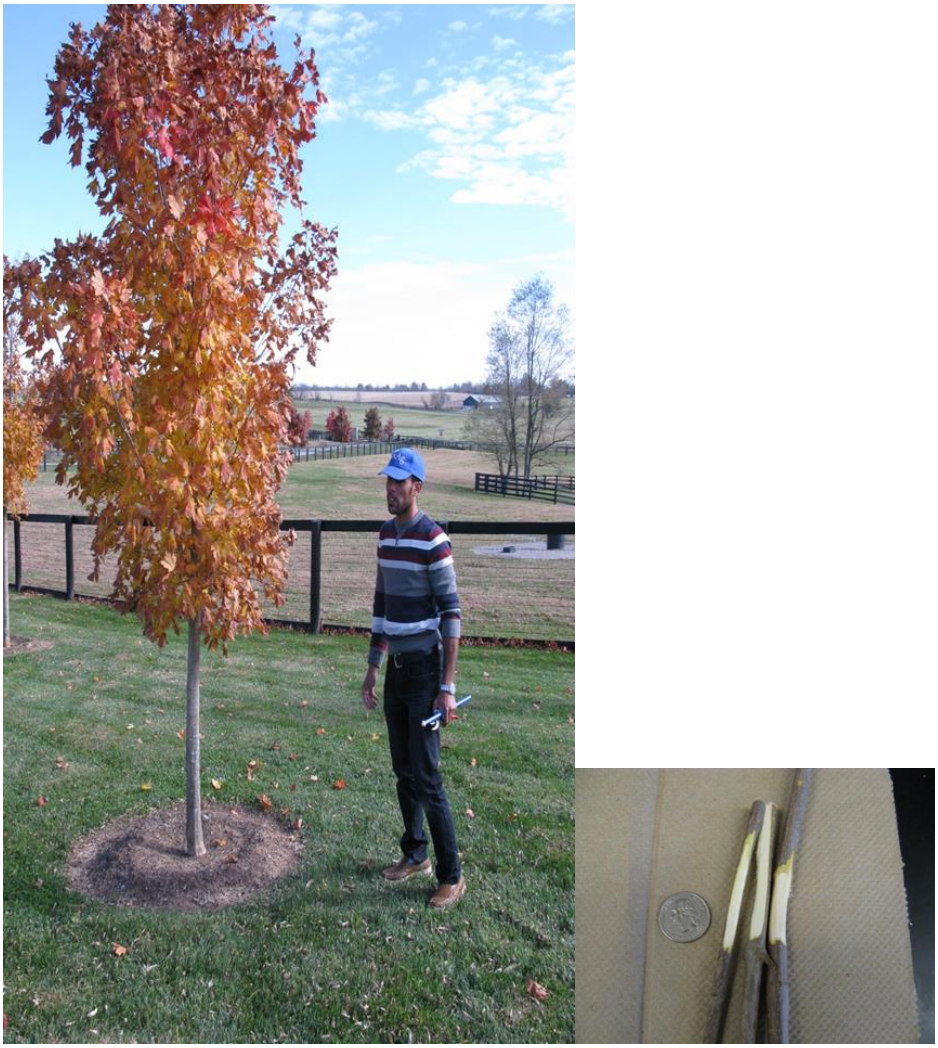


Figure 2.5. A bead beater (Biospec Products 3110BX) used to pulverize the shavings of woody tissues before extracting DNA.

(1) **(2)**

Figure 2.6. (1) Sugar maple tree exhibiting wilt, chlorosis, epinasty, and stunting, but without vascular discoloration, and (2) its twigs showing no vascular discoloration, Sampled at Spy Coast Farm in Lexington, Fayette Co, Kentucky.

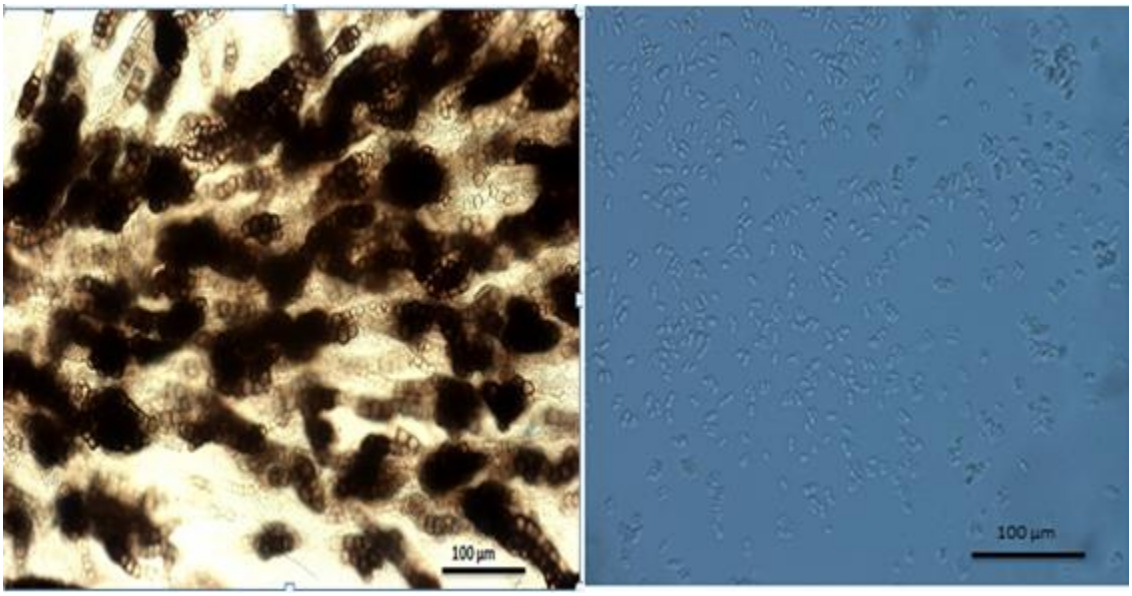


(1) **(2)**

Figure 2.7. Asymptomatic sugar maple showing only normal fall coloration (1), including an absence of vascular discoloration (2). Sampled at Spy Coast Farm in Lexington, Fayette Co, Kentucky.

(1) **(2)**

Figure 2.8. Five-year-old symptomatic smoke trees (*Cotinus coggygria*) with wilt and with vascular discoloration, sampled from Frankfort, Kentucky.



(1) **(2)**

Figure 2.9. Morphological features of *V. dahliae* after 12 days of growth at room temperature. (1) Microsclerotia of *V. dahliae* on potato dextrose agar (PDA). (2) Conidia of *V. dahliae* on Czapek-Dox agar media.

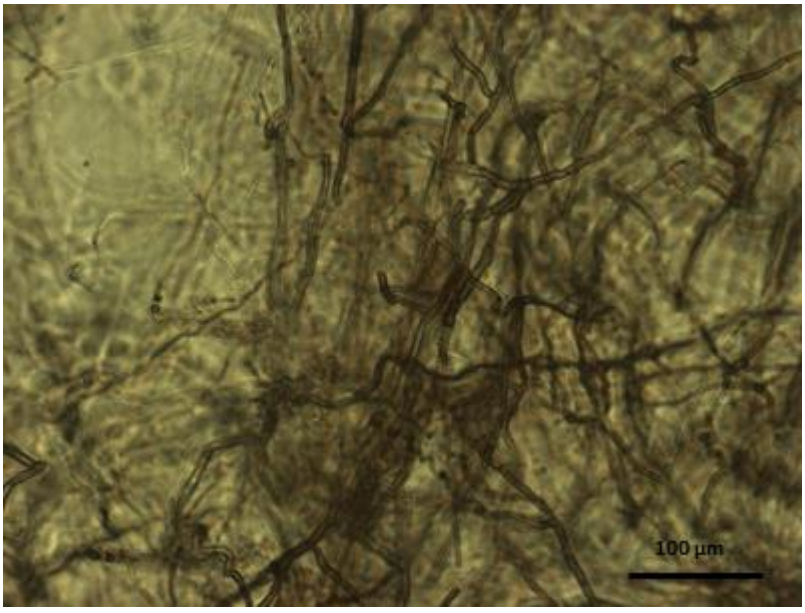
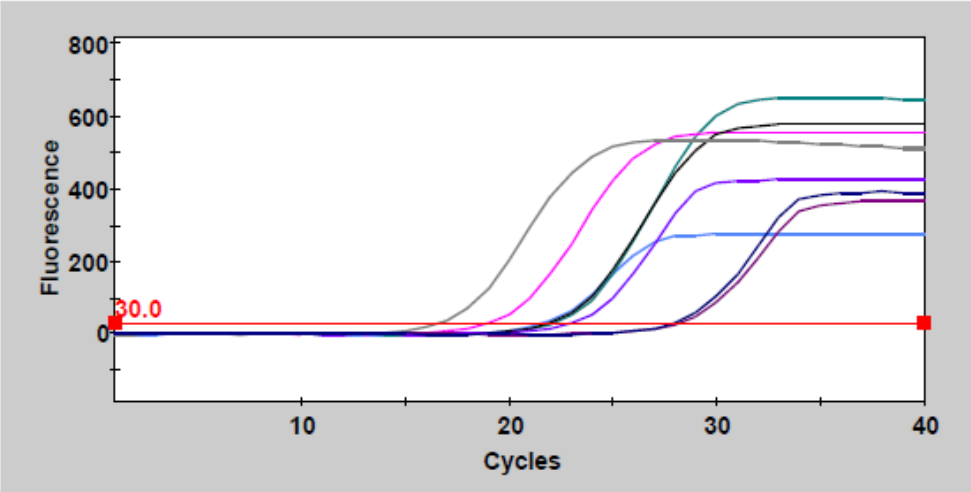


Figure 2.10. Production of melanized hyphae of *V. albo-atrum* on potato dextrose agar (PDA) after 12 days at the room temperature in the dark.



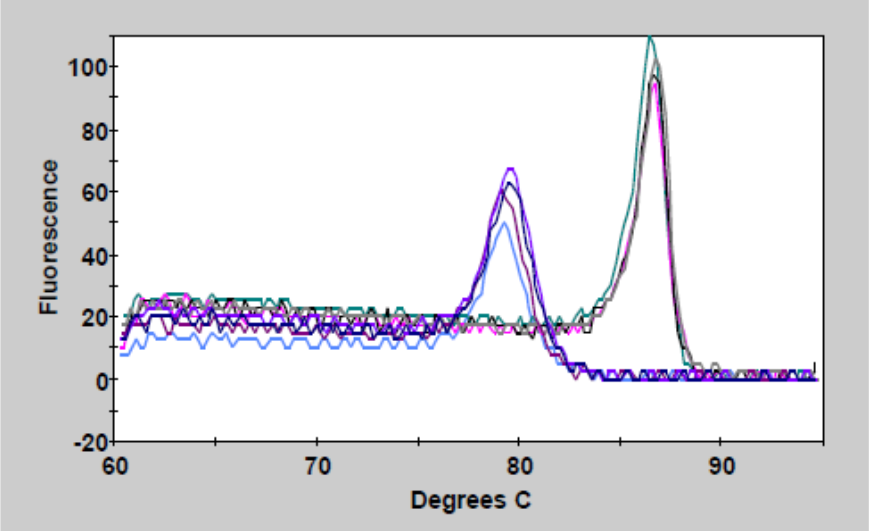
Site Legend

Site ID	Sample ID	Intcltr Ct	Protocol
A9	NPW	21.63	vert-vertBt
A10	V. dahliae(#64114)	22.15	vert-vertBt
A11	V. dahliae (#891)	19.10	vert-vertBt
A12	V. dahliae (#884)	21.94	vert-vertBt
A13	V.dahliae (#876)	16.68	vert-vertBt
A14	V. albo-atrum	28.23	vert-vertBt
A15	Phytopthora capsici	23.09	vert-vertBt
A16	Pyriwlria oryzae	28.04	vert-vertBt

Data Type	Line Type
Primary Curve	———

Channel	Symbol
Intcltr	None

Figure 2.11. PCR amplification curves for *V. dahliae* in a SYBR® Green-based assay using the VertBt primer set.



Site Legend

Site ID	Sample ID	Melt Peak1	Melt Peak2	Protocol
A9	NPW	79.28	77.19	vert-vertBt
A10	V. dahliae(#64114)	86.47	62.72	vert-vertBt
A11	V. dahliae (#891)	86.65	63.53	vert-vertBt
A12	V. dahliae (#884)	86.7	62.78	vert-vertBt
A13	V.dahliae (#876)	86.79	84.45	vert-vertBt
A14	V. albo-atrum	79.1	77.1	vert-vertBt
A15	P. capsici	79.56	77.36	vert-vertBt
A16	P. oryzae	79.52	64.86	vert-vertBt

Data Type	Line Type
1st Derivative	

Figure 2.12. Melt derivative curves show specific melt temperatures (melt Peak1) which were generated from PCR amplicons in a SYBR® Green-based assay using VertBt primer set for detecting *V. dahliae***.**

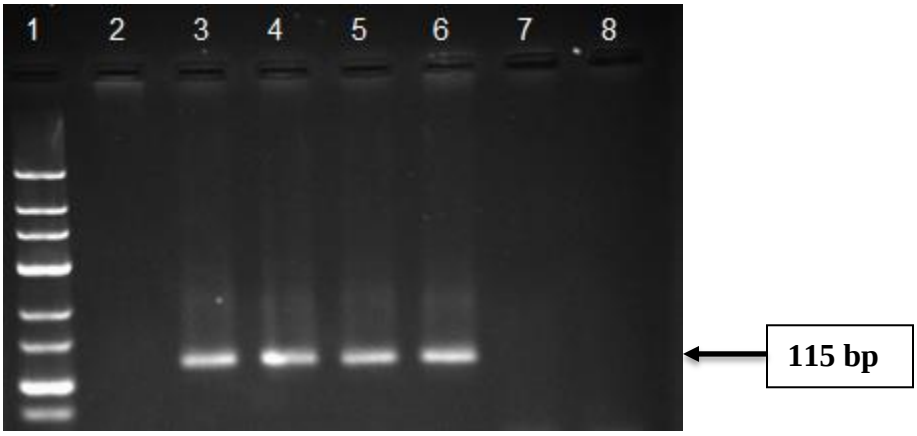
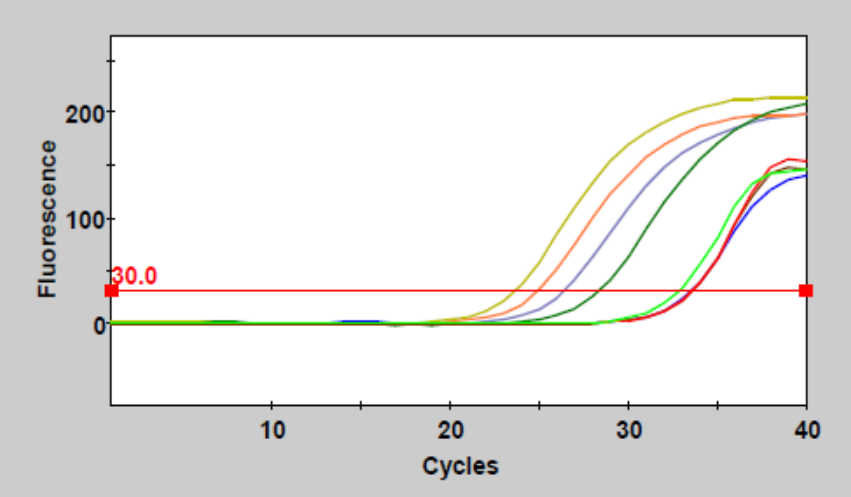


Figure 2.13. Ethidium bromide-stained 2% (w/v) agarose gel containing amplified products from PCR amplification with primers VertBt-F and VertBt-R. Lane 1: 100 bp O'Gene Ruler LR DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: *P. capsici*. DNA of *P. oryzae* produced no amplicon (*data not shown*).



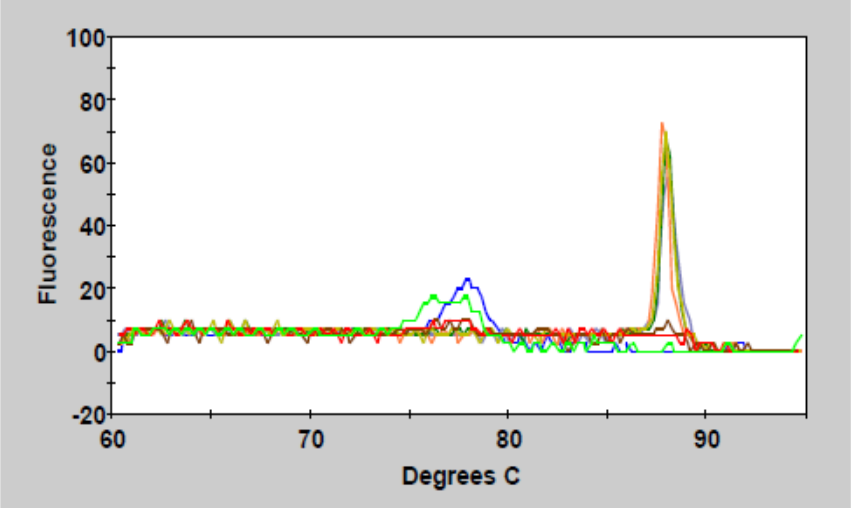
Site Legend

Site ID	Sample ID	Intcltr Ct	Protocol
A1	NPW	33.43	vert-VDS
A2	V. dahliae (#64114)	26.37	vert-VDS
A3	V. dahliae (#891)	24.86	vert-VDS
A4	V. dahliae (#884)	28.32	vert-VDS
A5	V. dahliae (#876)	23.56	vert-VDS
A6	V. albo-atrum	33.53	vert-VDS
A7	P. capsici	33.52	vert-VDS
A8	P. oryzae	32.77	vert-VDS

Data Type	Line Type
Primary Curve	———

Channel	Symbol
Intcltr	None

Figure 2.14. PCR amplification curves generated for *V. dahliae* in a SYBR® Green-based assay using the VDS primer set.



Site Legend

Site ID	Sample ID	Melt Peak1	Melt Peak2	Protocol
A1	NPW	77.93	77.51	vert-VDS
A2	V. dahliae (#64114)	88.05	87.19	vert-VDS
A3	V. dahliae (#891)	87.79		vert-VDS
A4	V. dahliae (#884)	87.97	62.55	vert-VDS
A5	V. dahliae (#876)	87.93	65.09	vert-VDS
A6	V. albo-atrum	77.72	76.34	vert-VDS
A7	P. capsici	76.91	65.85	vert-VDS
A8	P. oryzae	76.23	77.85	vert-VDS

Data Type	Line Type
1st Derivative	———

Figure 2.15. Melt derivative show specific melt temperatures (melt Peak1) generated from PCR amplicons in a SYBR® Green-based assay using the VDS primer set for detecting *V. dahliae*.

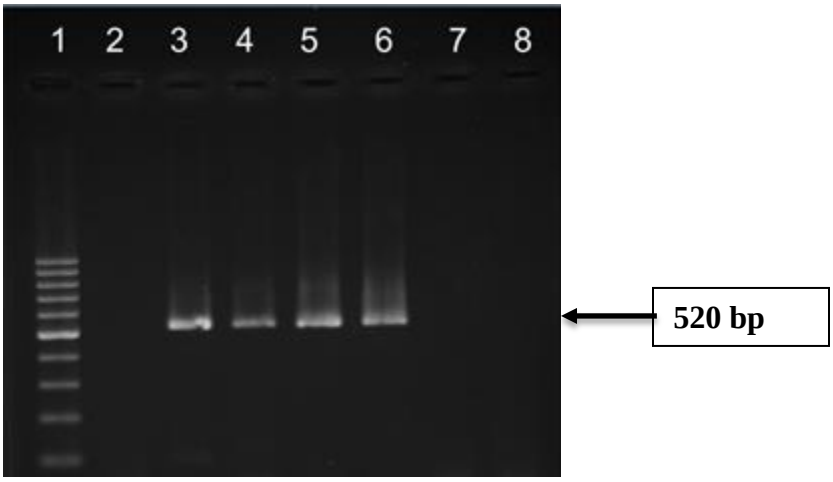


Figure 2.16. Ethidium bromide-stained 1.5% (w/v) agarose gel containing amplified products from PCR amplification with primers VDS1 and VDS2. Lane 1: 100 bp O'Gene Ruler DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: *P. capsici*. DNA of *P. oryzae* produced no amplicon (*data not shown*).

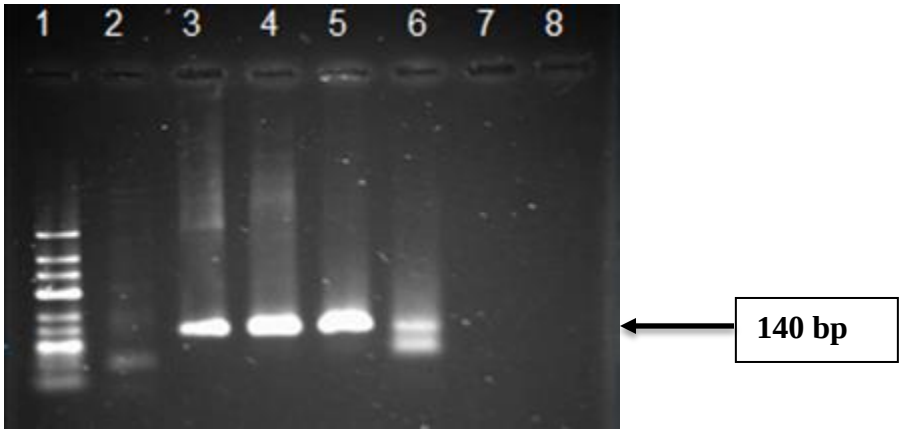


Figure 2.17. Ethidium bromide-stained 1.5% (w/v) agarose gel containing amplified products from PCR amplification with primers VMSP1 and VMSP2. Lane 1: 100 bp O'Gene DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: *P. capsici*. DNA of *P. oryzae* produced no amplicon (*data not shown*).

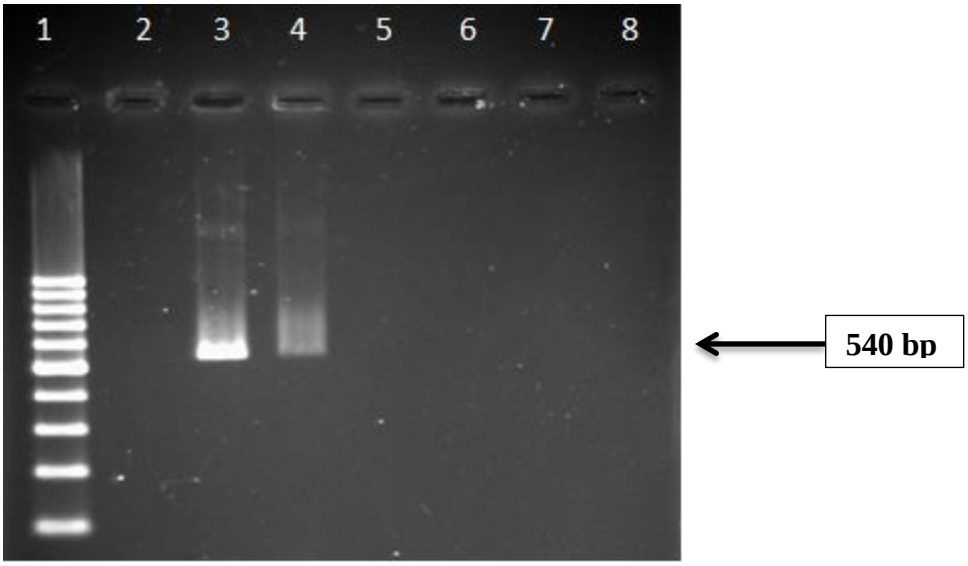


Figure 2.18. Ethidium bromide-stained 2% (w/v) agarose gel containing amplified products from PCR amplification with primers DB19/DB22, Lane 1: O'Gene DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: *P. capsici*. DNA of *P. oryzae* produced no amplicon (*data not shown*).

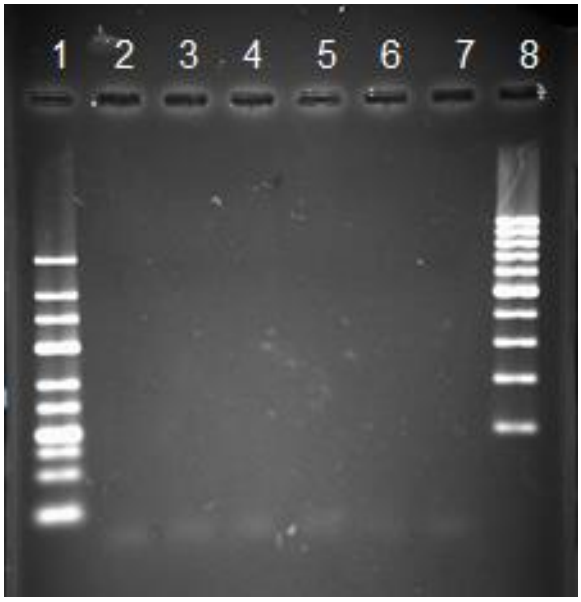


Figure 2.19. Ethidium bromide-stained 2% (w/v) agarose gel showing no amplification products from PCR amplification with primers Vd-F929-947/Vd-R1076 with corresponding Taqman® probe, Lane 1: O'Gene LR DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: 100 O'Gene DNA ladder. DNA of *P. oryzae* produced no amplicon (*data not shown*).

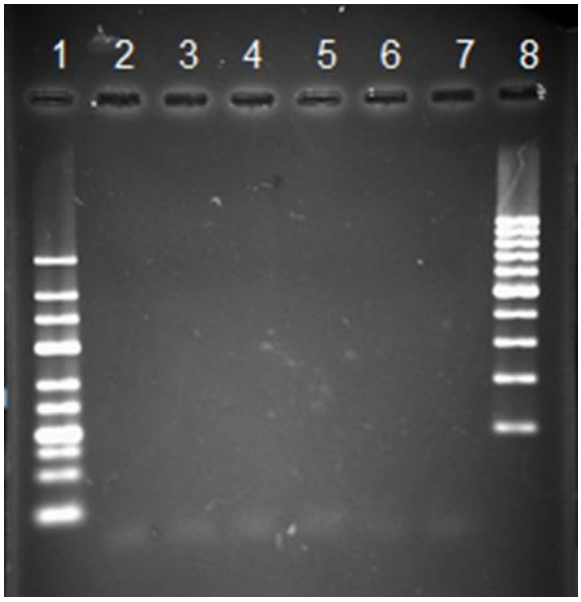


Figure 2.20. Ethidium bromide-stained 2% (w/v) agarose gel showing no amplification products from PCR amplification with VTP1-2F/VTP1-2R with corresponding Taqman® probe. Lane 1: O'Gene LR DNA Ladder Plus; lane 2: sterile distilled water; lanes 3, 4, 5 and 6: *V. dahliae* isolates (#891, #884, #876, and #64114); lane 7: *V. albo-atrum*; lane 8: 100 O'Gene DNA ladder. DNA of *P. oryzae* produced no amplicon (*data not shown*).

VMSP2

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Vd_G12.1.   : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_NOVA2.   : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_NOVA3.   : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_76.      : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_T9.      : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_M5.      : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_UZ132.   : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vd_k12.     : ATAGGTTTAAAGGGTACTCAAACGGTCAAATTATCCTTTAA :   41
Vd_AFTOL.   : ATAGGTTTAAAGGGTACTCAAACGGTCAGATTATCCTTTAA :   41
Vaa_1776.   : ATAGGTTTAAAGGGTACTCAAACGGTCAAATTATCCTTTAA :   41
Vaa_CBS7.   : ATAGGTTTAAAGGGTACTCAAACGGTCAAATTATCCTTTAA :   41
Vn_IMI_27.  : ATAGGTTTAAAGGGTACTCAAACGGTCAAATTATCCTTTAA :   41
Vf_IMI_18.  : TTAGGTTTAAAGGGTACTCAAACGGTCA------------ :   28
Vni_71799.  : TTAGGTTTAAAGGGTACTCAAACGGTCAAGATATCCTATAA :   41
Vth_IMI_2.  : TTAGGTTTAAAGGGTACTCAAACGGTCAAAATATCCGA-AC :   40
Vt_188.     : ATAGGTTTAAAGGGTACTCAAACGGTCAAATTATCCTTTAA :   41
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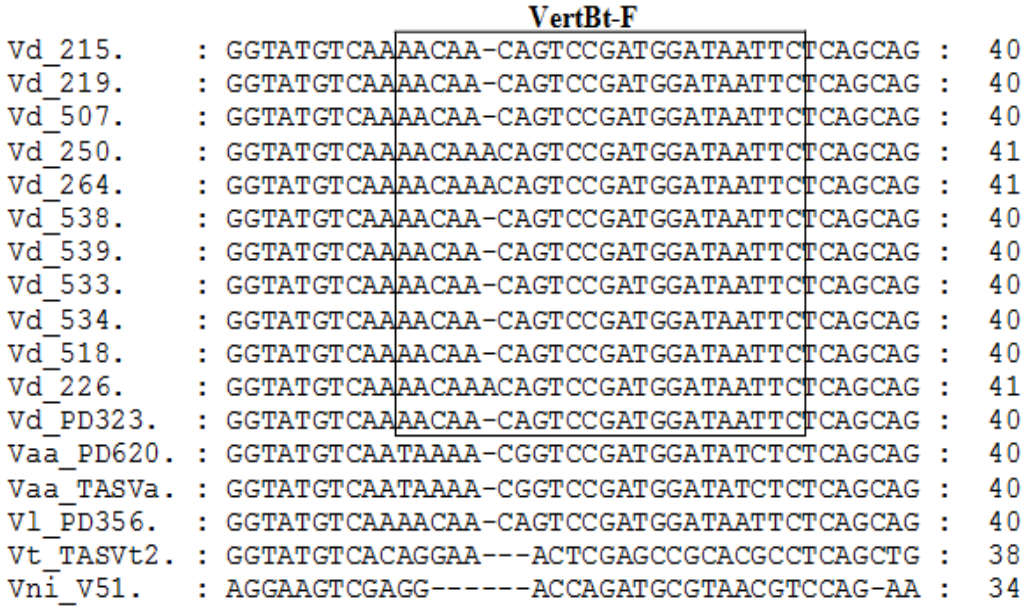
Vd_G12.1.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_NOVA2.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_NOVA3.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_76.      : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_T9.      : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_M5.      : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_UZ132.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_k12.     : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vd_AFTOL.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vaa_1776.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vaa_CBS7.   : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vn_IMI_27.  : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vf_IMI_18.  : ---GTAAAAC--GACTAGAGTTATATAGAAGAAGGCAGTAC :  64
Vni_71799.  : AAGGTAATACTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
Vth_IMI_2.  : CAGGTAATACTTGACTAGAGTTTTATATAAGAAGACAGAAC :  81
Vt_188.     : TAGGTAATATTTGTCTAGAGTTTTATGTAAGAAGACAGTAC :  82
```

```
Vd_G12.1.   : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_NOVA2.   : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_NOVA3.   : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_76.      : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_T9.      : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_M5.      : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_UZ132.   : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vd_k12.     : TTAGAGTGGAGAGATTAAATTCTGTGATACCTAAGGGACTG : 123
Vd_AFTOL.   : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123
Vaa_1776.   : TTAGAGTGGAGAGATTAAATTCTGTGATACCAAAGGGACTG : 123
Vaa_CBS7.   : TTAGAGTGGAGAGATTAAATTCTGTGATACCAAAGGGACTG : 123
Vn_IMI_27.  : TTAGAGTGGAGAGATTAAATTCTGTGATACCAAAGGGACTG : 123
Vf_IMI_18.  : CTTAAGTGTAGAGATTAAATGCTGTCATACTGGAGGGACTC : 105
Vni_71799.  : TTAGAGTGGAGAGATGTAATTCGTTGATACCAAAGGGACTG : 123
Vth_IMI_2.  : CTAGAGTGGAGAGATAAAATTCGTTGATACCAAGGGGACTG : 122
Vt_188.     : TTAGAGTGGAGAGATTATATTCTGTGATACCAAAGGGACTG : 123

                                VMSP1
Vd_G12.1.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_NOVA2.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_NOVA3.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_76.      : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_T9.      : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_M5.      : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_UZ132.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vd_k12._    : -GCTTCGGCGTAGGCAGTGTTTTATGTAAAAAC : 155
Vd_AFTOL.   :  -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vaa_1776.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vaa_CBS7.   : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vn_IMI_27.  : -GCAACGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
Vf_IMI_18.  : TGAAAAGGCGTAGGCAGCCTTCTAAGTAATAAC : 138
Vni_71799.  : -ATAATTGCGAAGGCAGTCTTTTATGTAATAAC : 155
Vth_IMI_2.  : -GTAAAGGCGAAGGCAGTCTTTTATGTAAAAAC : 154
Vt_188.     : -GCAATGGCGTAGGCAGTCTTTTATGTAAAAAC : 155
```

Figure 2.21. Nucleotide sequences alignment (5' to 3', Clustal W) generated from mitochondrion genome sequences of nine strains of *Verticillium dahliae* (Vd), two strains of *Verticillium albo-atrum* (Vaa), one strain of *Verticillium nubilum* (Vn), one strain of *Verticillium fungicola* (Vf), one strain of *Verticillium nigrescens* (Vni), one strain of *Verticillium theobromae* (Vth), and one strain of *Verticillium tricorpus* (Vt), downloaded from Genbank. The nucleotide sequences of primers VSMP1and VSMP2 are indicated on the alignment.




```
Vd_215.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_219.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_507.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_250.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  81
Vd_264.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  81
Vd_538.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_539.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_533.    : CATTTGCTCATGGTTTTCTTTCTTTG~CAGGCCTCTGGCAA :  80
Vd_534.    : CATTTGCTCATGGTTTTCTTTCTTTG-CAGGCCTCTGGCAA :  80
Vd_518.    : CATTTGCTCATGGTTTTCTTTCTTTG-CAGGCCTCTGGCAA :  80
Vd_226.    : CATTTGCTCATGGTTTTCTTTCTTTG-CAGGCCTCTGGCAA :  81
Vd_PD323.  : CATTTGCTCATGGTTTTCTTTCTTTG-CAGGCCTCTGGCAA :  80
Vaa_PD620. : CATTTGCTCATGGCTTTTTTTTTGTGGCAGGCCTCTGGCAA :  81
Vaa_TASVa. : CATTTGCTCATGGCTTTTTTTTTGTGGCAGGCCTCTGGCAA :  81
Vl_PD356.  : CATTTGCTCATGGTTTTCTTTCTTTG-CAGGCCTCTGGCAA :  80
Vt_TASVt2. : CACCCACTCACAAATCGAATA------CAGGCTTCCGGAAA :  73
Vni_V51.   : CAAGAACTCGT--CCTACTTCGTCGAGTGGATTCCCAACAA :  73
```

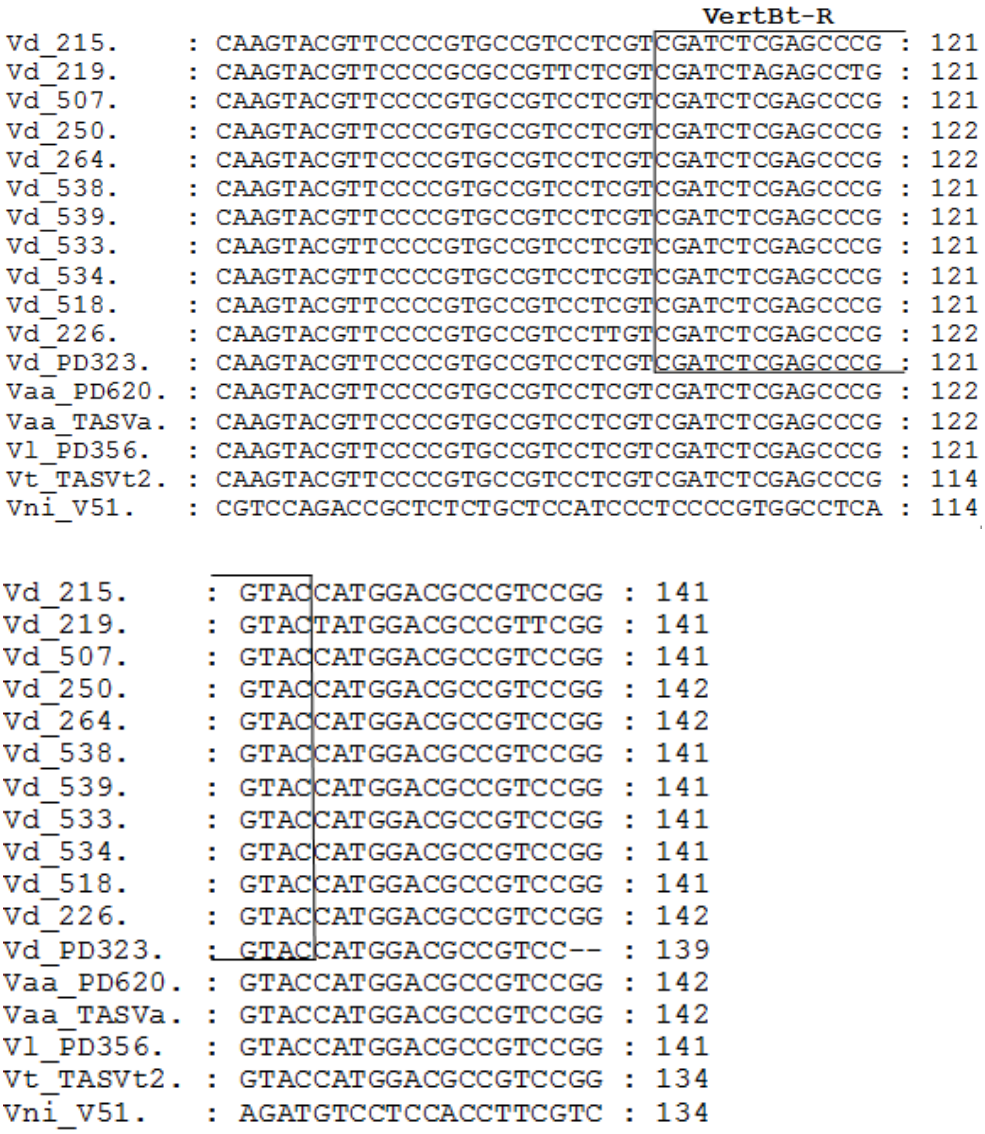
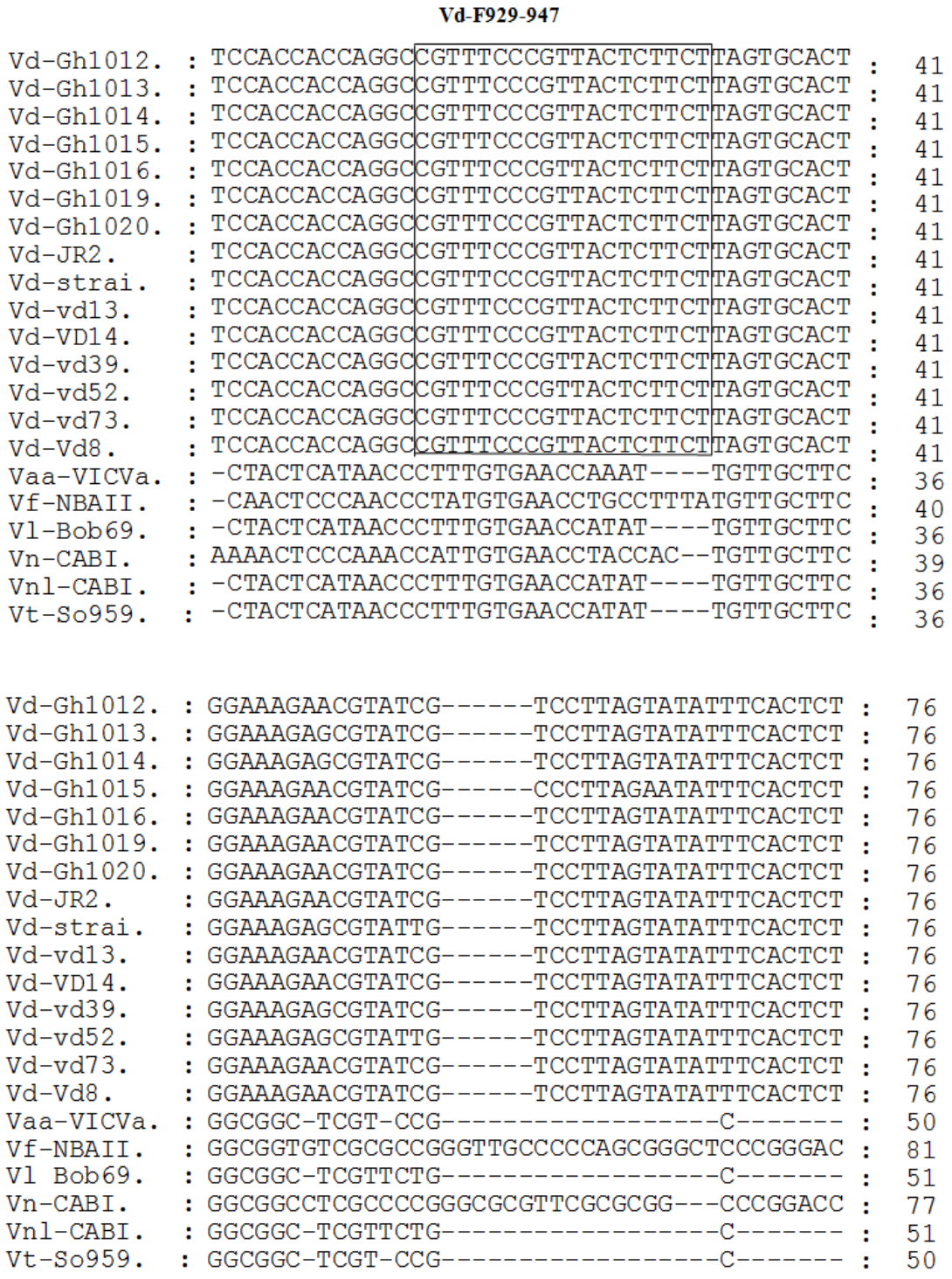


Figure 2.22**.** A portion of the βeta-tubulin gene of 12 strains of *Verticillium dahliae* (Vd), two strains of *Verticillium albo-atrum* (Vaa), one strain of *Verticillium longisporum* (Vl), one strain of *Verticillium tricorpus* (Vt), and one strain of *Verticillium nigrescens* (Vni), downloaded from the NCBI database. All sequences were aligned using Clustal W in the Bioedit sequence alignment editor program, and forward and reverse primers (VertB-F/VertB-R) are indicated on the alignment.


Vd-F929-947
Vd-Gh1012. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1013. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1014. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1015. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1016. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1019. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Gh1020. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-JR2. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-strai. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-vd13. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-VD14. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-vd39. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-vd52. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-vd73. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vd-Vd8. : TCCACCACCAGGCCGTTTCCCGTTACTCTTCTTAGTGCACT : 41
Vaa-VICVa. : -CTACTCATAACCCTTTGTGAACCAAAT----TGTTGCTTC : 36
Vf-NBAII. : -CAACTCCCAACCCTATGTGAACCTGCCTTTATGTTGCTTC : 40
Vl-Bob69. : -CTACTCATAACCCTTTGTGAACCATAT----TGTTGCTTC : 36
Vn-CABI. : AAAACTCCCAAACCATTGTGAACCTACCAC--TGTTGCTTC : 39
Vnl-CABI. : -CTACTCATAACCCTTTGTGAACCATAT----TGTTGCTTC : 36
Vt-So959. : -CTACTCATAACCCTTTGTGAACCATAT----TGTTGCTTC : 36
Vd-Gh1012. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Gh1013. : GGAAAGAGCGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Gh1014. : GGAAAGAGCGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Gh1015. : GGAAAGAACGTATCG------CCCTTAGAATATTTCACTCT : 76
Vd-Gh1016. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Gh1019. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Gh1020. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-JR2. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-strai. : GGAAAGAGCGTATTG------TCCTTAGTATATTTCACTCT : 76
Vd-vd13. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-VD14. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-vd39. : GGAAAGAGCGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-vd52. : GGAAAGAGCGTATTG------TCCTTAGTATATTTCACTCT : 76
Vd-vd73. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vd-Vd8. : GGAAAGAACGTATCG------TCCTTAGTATATTTCACTCT : 76
Vaa-VICVa. : GGCGGC-TCGT-CCG----------C------- : 50
Vf-NBAII. : GGCGGTGTCGCGCCGGGTTGCCCCCAGCGGGCTCCCGGGAC : 81
Vl Bob69. : GGCGGC-TCGTTCTG----------C------- : 51
Vn-CABI. : GGCGGCCTCGCCCCGGGCGCGTTCGCGCGG---CCCGGACC : 77
Vnl-CABI. : GGCGGC-TCGTTCTG----------C------- : 51
Vt-So959. : GGCGGC-TCGT-CCG----------C------- : 50

```
Vd-Gh1012.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1013.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1014.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1015.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1016.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1019.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Gh1020.  : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-JR2.     : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-stra.    : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-vd13.    : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-VD14.    : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-vd39.    : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-vd52.    : TAAAAGTACTATATATACTTGAACGCTATTTTGTTTTTGGC : 117
Vd-vd73.    : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vd-Vd8.     : TAAAAGTACTATATATACTTGAACGCTATTTG--TTTTGGC : 115
Vaa-VICVa.  : ---GAGCCCGCCGGT-ACATCAGT-CTCTATA-TTTTT---  :  82
Vf-NBAII.   : CACGCGTCCGCCGGAGACCAAAAA-CTCTTGA-TTTTGCGA  : 120
Vl-Bob69.   : ---GAGCCCGCCGGT-ACATCAGT-CTCTTTA-TTTAT---  :  83
Vn-CABI.    : CAGGCGTCCGCCGGAGGCTCCAAA-CTCTTGT-CTTT----  : 112
Vnl-CABI.   : ---GAGCCCGCCGGT-ACATCAGT-CTCTTTA-TTCAT---  :  83
Vt-So959.   : ---GAGCCCGCCGGT-ACATCAGT-CTCTTTA-TTTTT---  :  82
```

***V. dahliae* probe**

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Vd-Gh1012.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-Gh1013.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACTAGTATTC : 148
Vd-Gh1014.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACTAGTATTC : 148
Vd-Gh1015.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-Gh1016.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-Gh1019.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-Gh1020.  : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-JR2.     : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-strai.   : TTTCAAGAGTCTGCTTGCGGTGC--------ACTAGTATTC : 148
Vd-vd13.    : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-VD14.    : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-vd39.    : TTTCAAGAGTCTGCTTGCGGTGC--------ACTAGTATTC : 148
Vd-vd52.    : TTTCAAGAGTCTGCTTGCGGTGC--------ACTAGTATTC : 150
Vd-vd73.    : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vd-Vd8.     : TTTCAAGAGTCTGCTTGCGGTGC--------ACCAGTATTC : 148
Vaa-VICVa.  : ACCAACGATACTTCTGAGTGTTC---------TTA-CGAAC : 113
Vf-NBAII.   : AAGCAGTATTATTCTGAGTGGCCGAAAGGCAAAAAACAAAT : 161
Vl-Bob69.   : ACCAACGATACTTCTGAGTGTTC---------TTAGCGAAC : 115
Vn-CABI.    : ---TAGTGTATTTCTGAGTGGC---------ATAAGCAAAT : 141
Vnl-CABI.   : ACCAACGATACTTCTGAGTGTTC---------TTAGCGAAC : 115
Vt-So959.   : ACCAACGATACATCTGAGTGTTC---------TTAGCGAAC : 114
```

Figure 2.23. An alignment of intragenic spacer IGS sequences of *Verticillium* spp. including 15 strains of *V. dahliae* (Vd), one strain of *V. albo-atrum* (Vaa), one strain of *V. fungicola* (Vf), one strain of *V. longisporum* (Vl), one strain of *V. nubilum* (Vn), one strain of *V. nigrescens* (VnI), and one strain of *V. tricorpus* (Vt). The alignment was generated using the Bioedit sequence alignment editor program after downloading all sequences from NCBI database. Primers Vd-F929-947 and Vd-R1076-1094, as well the probe, are indicated on the alignment.

DB19

```
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
V_dahliae_ : CGGTGACATAATACTGAGAGAGGAAGCATGCGACAGGAGCG :  41
```

```
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
V_dahliae_ : ATGAGCCCCTCGGTCGCGCCGACTTCGTCCCGAGCTCTGAA :  82
```

```
V_dahliae_ : GTAGTGGCAAGCGTGCATGTCGGCGAGTTTGGCATGTTTAG : 123
V_dahliae_ : GCAGTGGCAAGCGTGGATGTCGGCGAGTTTGGCATGTTTAG : 123
V_dahliae_ : GTAGTGGCAAGCGTGCATGTCGGCGAGTTTGGCATGTTTAG : 123
V_dahliae_ : GCAGTGGCAAGCGTGGATGTCGGCGAGTTTGGCATGTTTAG : 123
V_dahliae_ : GCAGTGGCAAGCGTGGATGTCGGCGAGTTTGGCATGTTTAG : 123
V_dahliae_ : GTAGTGGCAAGCGTGCATGTCGGCGAGTTTGGCATGTTTAG : 123
```

```
V_dahliae_ : CATCCCGCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTC : 164
V_dahliae_ : CATCCCGCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTC : 164
V_dahliae_ : CATCCCGCTCGTCATGTGTTTCGGCAACTCCAGCAAAGTTC : 164
V_dahliae_ : CATCCCGCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTC : 164
V_dahliae_ : CATCCCGCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTC : 164
V_dahliae_ : CATCCCGCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTC : 164
```

```
V_dahliae_ : CCCTGCACGGGTCCTGCAGCTTTCTGGTTCAGATGGGCGCG : 205
V_dahliae_ : CC-TGCACGGGTCCCGCAGCTTTCTGGTTCAGATGGGCGCG : 204
V_dahliae_ : CCCTGCACGGGTCCCGCAGCTTTCTGGTTCAGATGGGCGCG : 205
V_dahliae_ : CC-TGCACGGGTCCCGCAGCTTTCTGGTTCAGATGGGCGCG : 204
V_dahliae_ : CC-TGCACGGGTCCCGCAGCTTTCTGGTTCAGATGGGCGCG : 204
V_dahliae_ : CCCTGCACGGGTCCTGCAGCTTTCTGGTTCAGATGGGCGCG : 205
```

```
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACCATGTCCTCGA : 246
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACTATGTTCTCGA : 245
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACTATGTTCTCGA : 246
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACTATGTTCTCGA : 245
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACTATGTCCTCGA : 245
V_dahliae_ : GGCCTGAAGAATATGCGGCAGTCTATCGACCATGTCCTCGA : 246
```

```
V_dahliae_ : GGGAGGCTTAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 287
V_dahliae_ : GGGAGGCTCAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 286
V_dahliae_ : GGGAGGCTCAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 287
V_dahliae_ : GGGAGGCTCAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 286
V_dahliae_ : GGGAGGCTCAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 286
V_dahliae_ : GGGAGGCTTAAGTTAACTACGGCACTAAAGGGTCAGCCAGG : 287
```

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                                                    espdfo1
V_dahliae_ : TATGAGGTCCATATCCAACACGAGCTGGAGCGTGTGGCAGC : 328
V_dahliae_ : TATGAGGTCCATATCCAACACAAGCTGGAGCGTGTGGCGGC : 327
V_dahliae_ : TATAAGGTCCATATCCAACACGAGCTGGAG---------- : 317
V_dahliae_ : TATAAGGTCCATATCCAACACGAGCTGGAG---------- : 316
V_dahliae_ : TATGAGGTCCATATCCAACACGAGCTGGAG---------- : 316
V_dahliae_ : TATGAGGTCCATATCCAACACGAGCTGGAGCGTGTGGCAGC : 328
```

```
V_dahliae_ : CGAGTCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 369
V_dahliae_ : CGAGTCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 368
V_dahliae_ : ----TCTCACCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 354
V_dahliae_ : ----TCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 353
V_dahliae_ : ----TCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 353
V_dahliae_ : CGAGTCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG : 369
```

```
V_dahliae_ : TATCTTATATACATGACAGCGATGAGACTGTCGAGCACCTC : 410
V_dahliae_ : TATCTTATATACATGACAGCGATGAGACTGTCGAGCACCTC : 409
V_dahliae_ : TATCTTATATACATGACAGCGATGTGACTGTCGAGCACCTC : 395
V_dahliae_ : TATCTTATATACATGACAGCGATGTGACTGTCGAGCACCTC : 394
V_dahliae_ : TATCTTATATACATGACAGCGATGAGACTGTCGAGCACCTC : 394
V_dahliae_ : TATCTTATATACATGACAGCGATGAGACTGTCGAGCACCTC : 410
```

Figure 2.24. Nucleotide sequences alignment generated from specific sequence of six strains of *V. dahliae*, downloaded from NCBI database. The nucleotide sequences of PCR primers DB19, DB22, and probe (espdfo1) are indicated on the alignment.

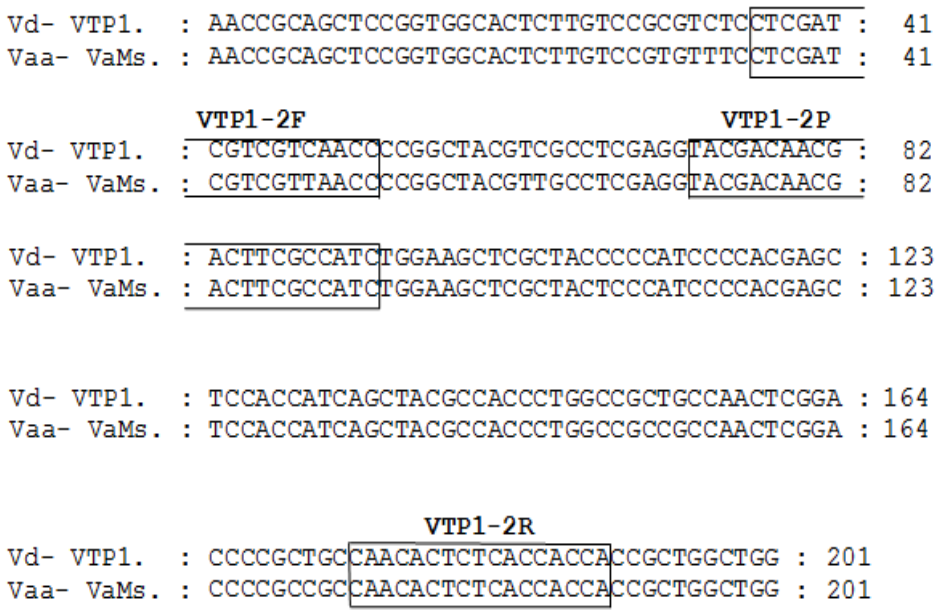

Figure 2.25. Sequence alignment of *TRYPSIN PROTEASE GENE* (*VTP1*) gene from one strain of *V. dahliae* (Vd) and one strain of *V. albo-atrum* (Vaa). The boxed areas show the primer and probe regions that were used in real-time PCR for detection of *V. dahliae*.

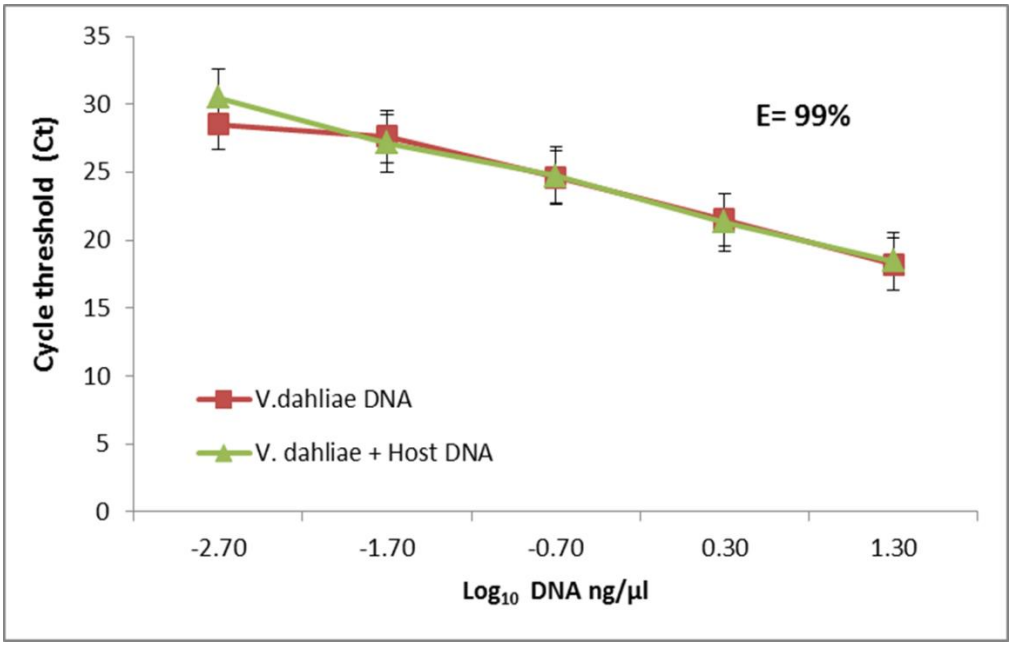

Figure 2.26. Real-time quantitative polymerase chain reaction (Q-PCR) efficiency curves using primers VertBt-F/VertBt-R and serially diluted genomic DNA of *V. dahliae* (isolate #884) in either the presence or the absence of DNA extracted from an uninfected maple tree. Bars indicate standard errors.

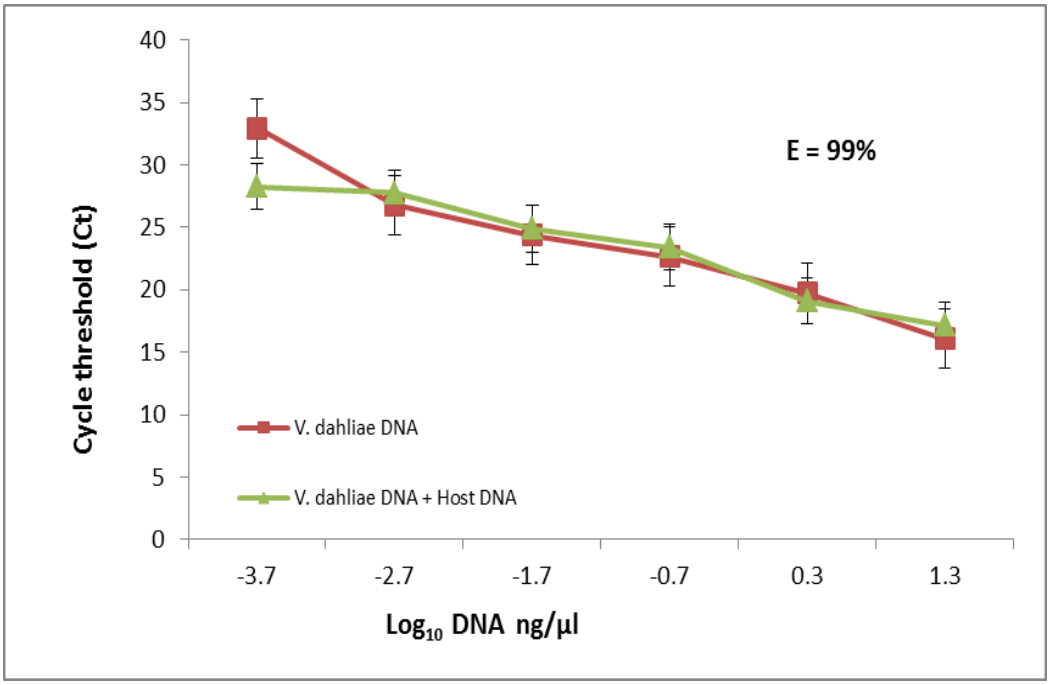

Figure 2.27. Real-time quantitative polymerase chain reaction (Q-PCR) efficiency curves using primers VDS1/VDS2 and serially diluted genomic DNA of *V. dahliae* (isolate #884) in either the presence or the absence of DNA extracted from an uninfected maple tree. Bars indicate standard errors.

(1) **(2)**

Figure 2.28. (1) Fine shavings were obtained from twigs by using an (1) APSCO pencil sharpener; or (2) Drill bits, 0.3 cm diameter.

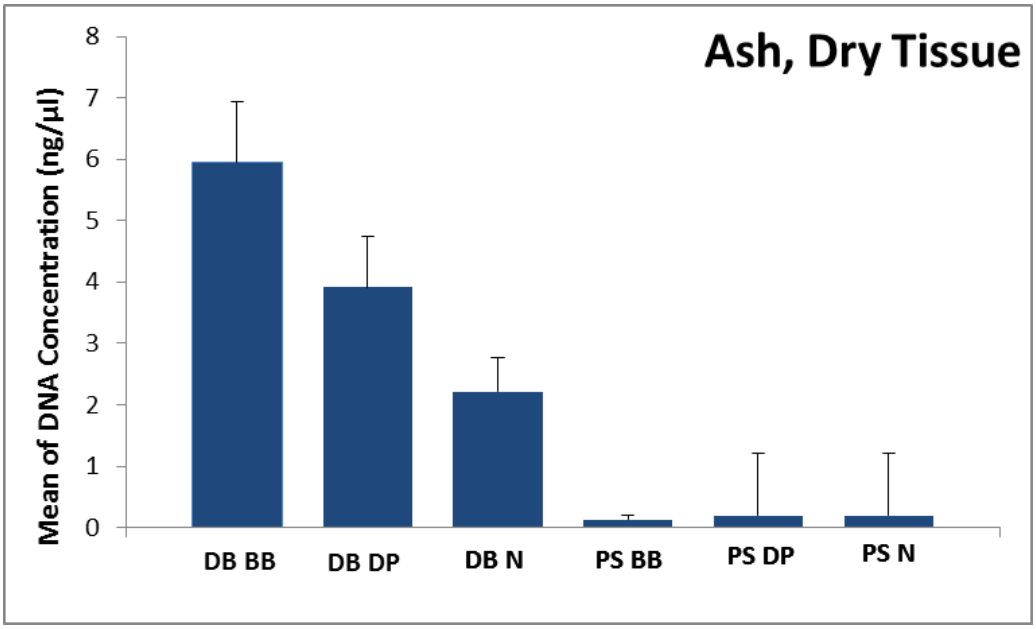

Figure 2.29. For oven-dried ash tissue, DNA yields from oven-dried samples processed by different methods of primary tissue disruption followed by secondary tissue disruption. Primary tissue disruption methods: DB, drill bit; PS, pencil sharpener. Secondary tissue disruption methods: BB, bead beater; DP, drill press with mesh bags; N, no secondary tissue disruption method. Bars indicate standard errors.

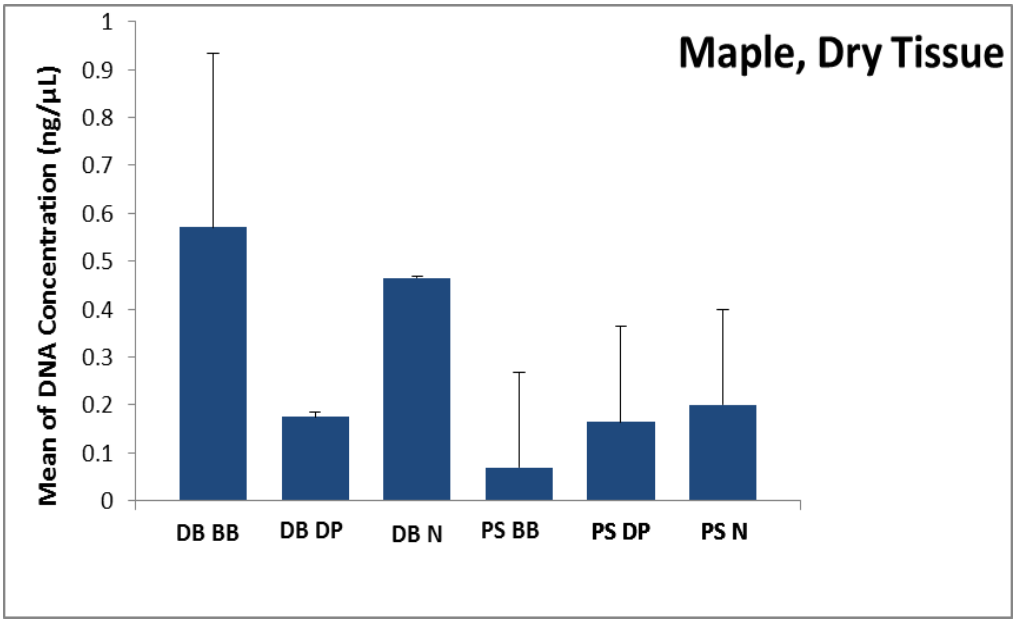

Figure 2.30. For oven-dried maple tissues, DNA yields from oven-dried samples processed by different methods of primary tissue disruption followed by secondary tissue disruption. Primary tissue disruption methods: DB, drill bit; PS, pencil sharpener. Secondary tissue disruption methods: BB, bead beater; DP, drill press with mesh bags; N, no secondary tissue disruption method. Bars indicate standard errors.

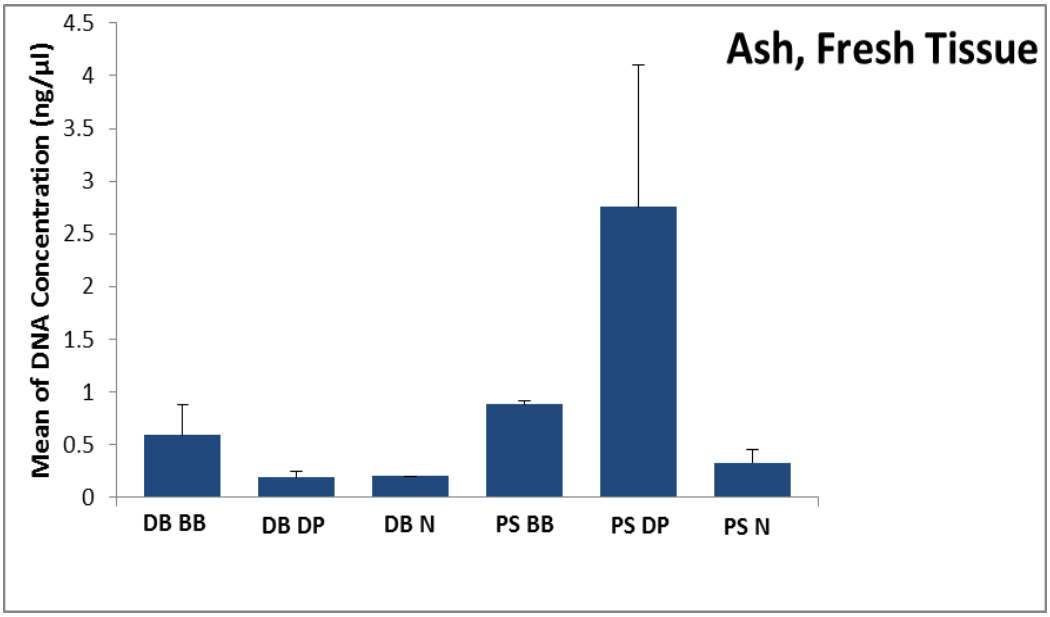

Figure 2.31. For fresh ash tissue, DNA yields from fresh samples processed by different methods of primary tissue disruption followed by secondary tissue disruption. Primary tissue disruption methods: DB, drill bit; PS, pencil sharpener. Secondary tissue disruption methods: BB, bead beater; DP, drill press with mesh bags; N, no secondary tissue disruption method. Bars indicate standard errors.

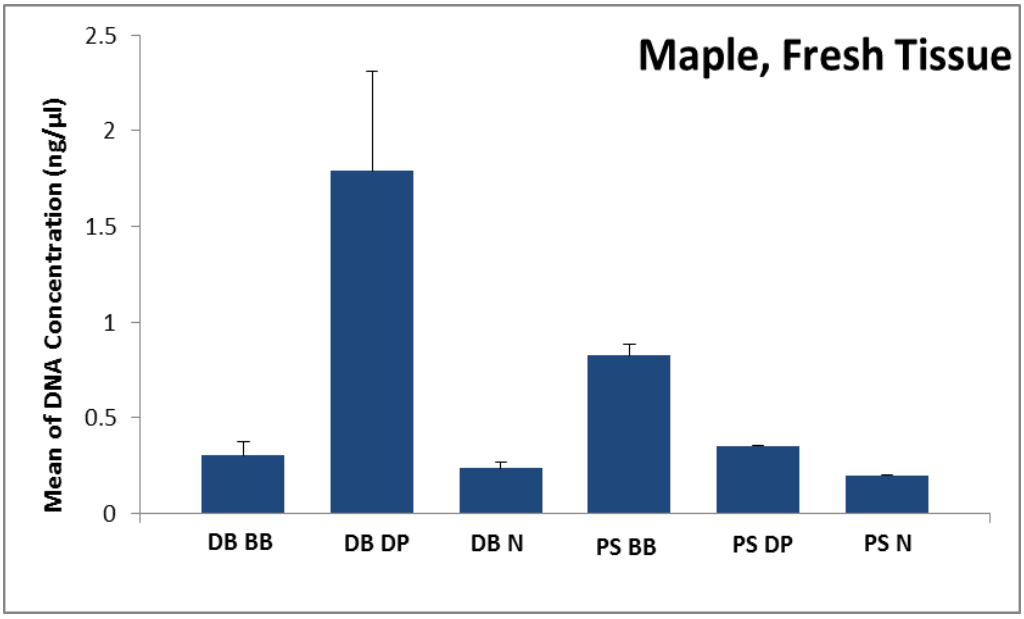

Figure 2.32. For fresh maple tissue, DNA yields from fresh samples processed by different methods of primary tissue disruption followed by secondary tissue disruption. Primary tissue disruption methods: DB, drill bit; PS, pencil sharpener. Secondary tissue disruption methods: BB, bead beater; DP, drill press with mesh bags; N, no secondary tissue disruption method. Bars indicate standard errors.

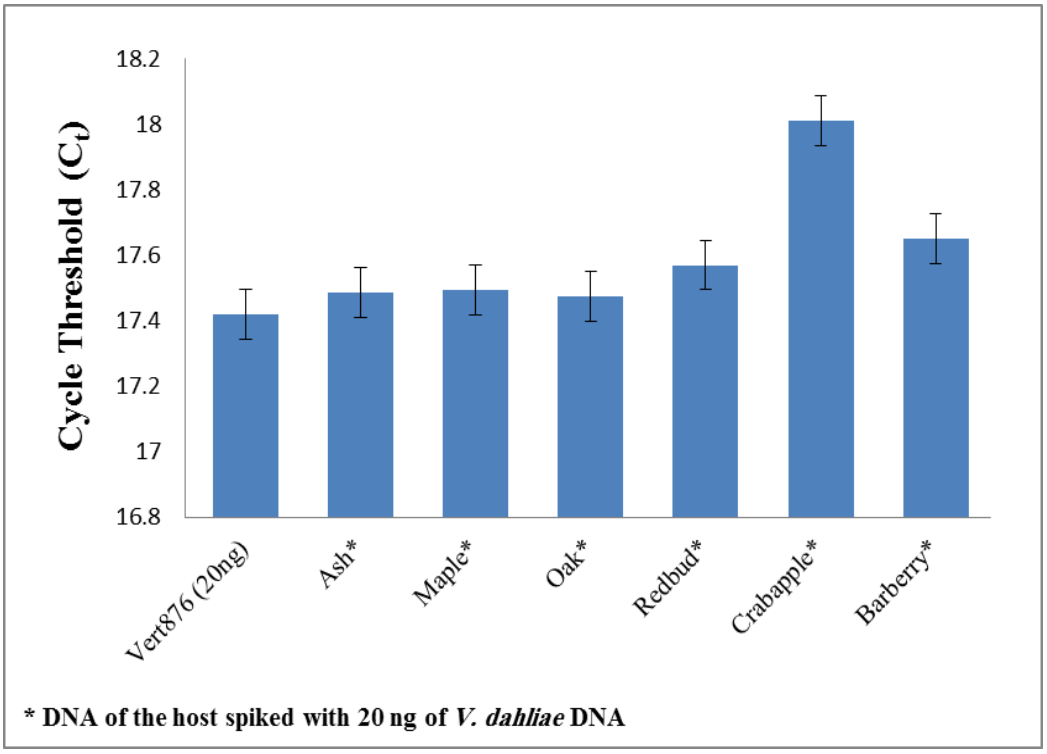

Figure 2.33. Effects of the presence of DNA extract from various woody species on real-time PCR using VertBt-F/VertBt-R primers for detection of *V. dahliae*. All PCR reactions received 20 ng extract of genomic DNA of *V. dahliae* isolate 876. Tubes marked with a plant name also received 20 ng of DNA extract obtain from asymptomatic, PCR-negative trees. Bars indicate standard errors.

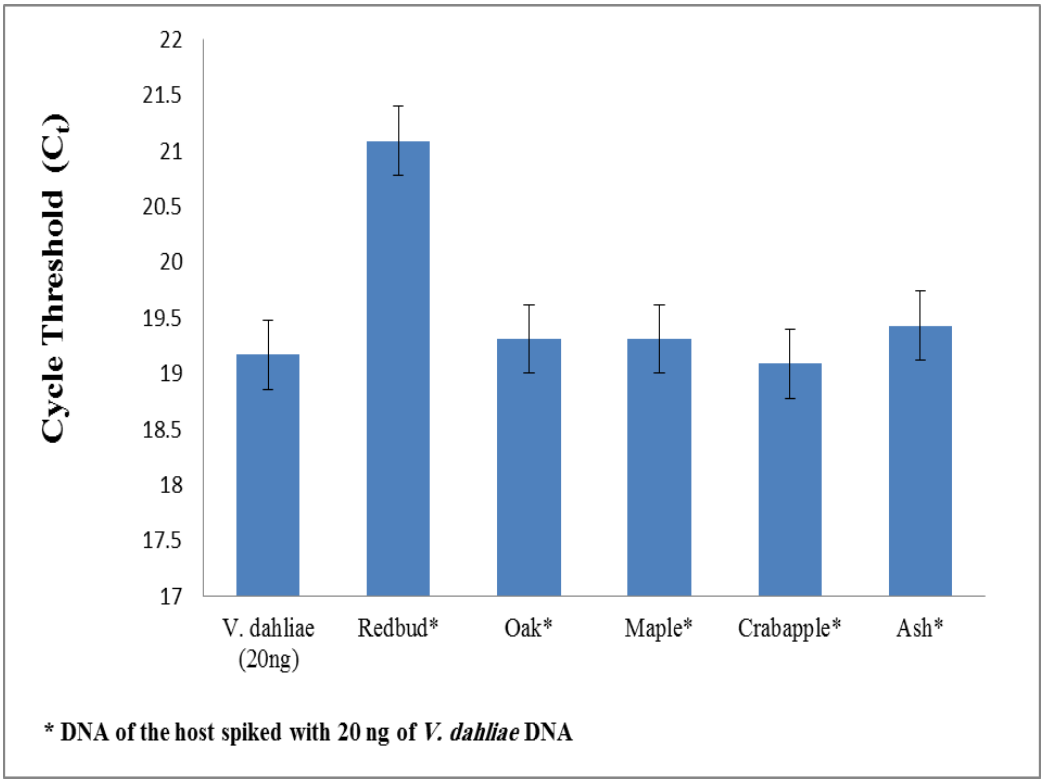

Figure 2.33. Effect of the presence of DNA extract from various woody species on real-time PCR using VDS primers for detection of *V. dahliae*. All PCR reactions received 20 ng extract of genomic DNA of *V. dahliae* isolate 876. Tubes marked with a plant name also received 20 ng of DNA extract obtain from asymptomatic, PCR-negative trees. Bars indicate standard errors.

Figure 2.34. Restriction sites identified using NEBcutter, version 1.0 (http://tools.neb.com/NEBcutter) for the expected amplicon generated by primers VertBt-F and VertBt-R.

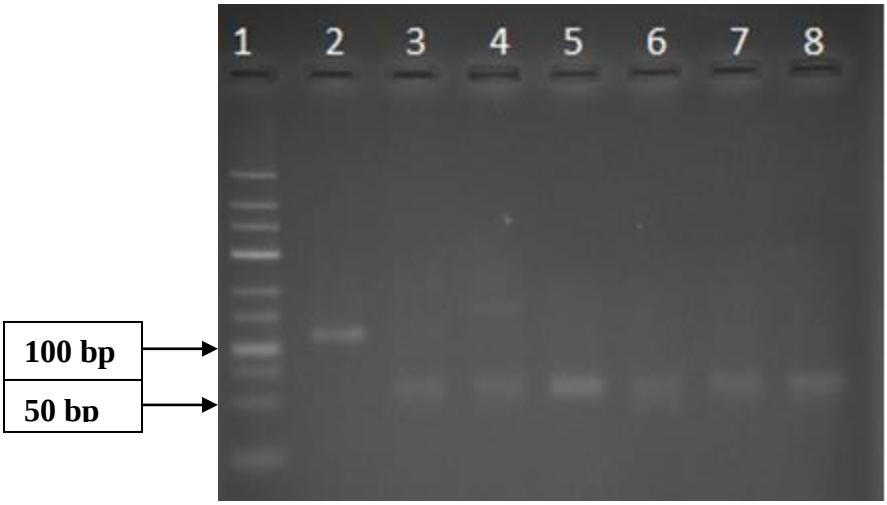

Figure 2.35. PCR amplicons of *V. dahliae* were generated with VertBt-F and VertBt-R primers, cleaved with restriction enzyme (HaeIII), and visualized by UV-fluorescence in 3% (w/v) Nusieve agarose gel after staining with ethidium bromide. Lane 1: 100 bp O'Gene LR DNA ladder marker; lane 2: uncut PCR amplicon of *V. dahliae* isolate # 891; remaining lanes are restriction digests of PCR amplicons from: lane 3-4, asymptomatic sugar maple trees (AST1 and AST2); lane 5-6: sugar maple trees exhibiting wilt but not vascular discoloration (WST1 and WST2); lane7-8: Smoke tree exhibiting both wilt and vascular discoloration (VST1 and VST2).

LITERATURE CITED

Al-Rawahi, A. K., & Hancock, J. G. (1998). Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. *Plant Disease, 82*(10), 1100-1106.

Al-Ahmad, M. A. (1993). The solar chamber: an innovative technique for controlling verticillium wilt of olive. *EPPO Bulletin (Organisation Europeenne et Mediterraneenne pour la Protection des Plantes) 23*(3), 531-535.

Almeida, R. F. (2009). *Host Transcriptional Responses to Vascular and Foliar Phytopathogenic Fungi* (Ph.D. Doctoral dissertation), BRASIL.

Arnheim, N., & Erlich, H. (1992). Polymerase chain reaction strategy. *Annual Review of Biochemistry, 61*(1), 131-156.

Ash, C. L. (1993). *Verticillium Wilt of Trees and Shrubs*. St. Paul, Minn.: University of Minnesota Extension Service.

Ash, C. L. (1999). *The Ecology of Verticillium dahliae in Fraxinus and Acer Species* (9929485 Ph.D.), University of Minnesota, Ann Arbor. ProQuest Dissertations & Theses Full Text database.

Ash, C. L. (2001, August 25-28). *Shade tree wilt diseases. Proceedings from Wilt Diseases of Shade Trees: a National Conference, St. Paul, Minnesota, USA, 25-28 August, 1999.* Paper presented at the Shade Tree Wilt

Diseases. Proceedings from Wilt Diseases of Shade Trees: a National Conference, St. Paul, Minnesota, USA, 25-28 August, 1999.

Atallah, Z. K., Bae, J., & Jansky, S. H. (2007). Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. *Phytopathology, 97*(7), 865-872.

Ausher, R. (1975). An improved selective medium for the isolation of *Verticillium dahliae*. *Phytoparasitica, 3*(2), 133-137.

Ausher, R., Katan, J., & Ovadio, S. (1975). An improved selective medium for the isolation of *Verticillium dahliae*. *Phytoparasitica, 3*(2), 133-137.

Banfield, W. M. (1941). Distribution by the sap stream of spores of three fungi that induce vascular wilt diseases of elm. *Journal of Agricultural Research, 62*, 637-681.

Bartlett, J. M., & Stirling, D. (2003). A short history of the polymerase chain reaction *PCR protocols* (pp. 3-6): Humana Press.

Beckman, C. H. (1987). *The Nature of Wilt Diseases of Plants*. St. Paul, Minnesota; USA: APS Press.

Ben-Yephet, Y., & Pinkas, Y. (1976). A cesium chloride flotation technique for the isolation of *Verticillium dahliae* microsclerotia from soil. *Phytopathology, 66*(10), 1252-1154.

Ben, C., Toueni, M., & Montanari, S. (2013). Natural diversity in the model legume Medicago truncatula allows identifying distinct genetic

mechanisms conferring partial resistance to Verticillium wilt. *Journal of Experimental Botany, 64*(1), 317-332.

Berg, G., Fritze, A., Roskot, N., & Smalla, K. (2001). Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. *Journal of applied microbiology, 91*(6), 963-971.

Berg, G., Knaape, C., & Ballin, G. (1994). Biological control of *Verticillium dahliae* Kleb. by natural occurring rhizosphere bacteria. *Archives of Phytopathology & Plant Protection, 29*(3), 249-262.

Bergeson, G. B. (1972). Concepts of nematode-Fungus associations in plant disease complexes. *A Review Experimental Parasitology, 32*(2), 301-314.

Berlanger, I. E. (1999). *Effect of a Broccoli Green Manure, Soil Solarization, and Isolates of Verticillium dahliae on Verticillium Wilt of Agronomic and Nursery Crops* (Master of Science), Oregon State University.

Bermingham, N., & Luettich, K. (2003). Polymerase chain reaction and its applications. *Current Diagnostic Pathology, 9*(3), 159-164.

Bessetti, J. (2007). An introduction to PCR inhibitors. *J. Microbiol. Meth, 28*, 159-167.

Bhat, R. G., & Subbarao, K. V. (1999). Host range specificity in *Verticillium dahliae*. *Phytopathology, 89*(12), 1218-1225.

Bilodeau, G. J., & Koike, S. T. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. *Phytopathology, 102*(3), 331-343.

Born, G. L. (1974). *Root Infection of Woody Hosts with Verticillium albo-atrum* (Vol. 31): State of Illinois, Department of Registration and Education.

Brandt, W. H. (1966). Influence of near-ultraviolet light on hyphal elongation in *Verticillium dahliae*. *Mycologia, 59*(4), 736-739.

Broders, K. D. (2010). Molecular Diagnostic Assay for Detection of the Butternut Canker Pathogen Sirococcus *clavigignenti-juglandacearum*. *Plant Disease, 94*(8), 952-958.

Bustin, S. (2005). Real-time PCR. *Encyclopedia of diagnostic genomics and proteomics, 10*, 1117-1125.

Butterfield, E. J., & DeVay, J. E. (1977). Reassessment of soil assays for *Verticillium dahliae*. *Phytopathology, 67*(8), 1073-1078.

Cernusko, R., & Wolf, G. A. (1997). Immunological methods for the detection of *Verticillium dahliae* in oilseed rape. In H. W. Dehne & G. Adam (Eds.), *Diagnosis and Identification of Plant Pathogens* (pp. 109-112). Germany: Kluwer Academic Publishers; Dordrecht; Netherlands.

Cha, R. S., & Thilly, W. G. (1993). Specificity, efficiency, and fidelity of PCR. *Genome Research, 3*(3), S18-S29.

Chalavi, V., & Tabaeizadeh, Z. (2003). Enhanced resistance to *Verticillium dahliae* in transgenic strawberry plants expressing a *Lycopersicon chilense* chitinase gene. *Journal of the Aamerican Society for Horticultural Science, 128*(5), 747-753.

Chen, W. (1994). Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. *Phytopathology, 84*(2), 214-219.

Cheo, P. C. (1982). Effects of tannic acid on rhizomorph production by *Armillaria mellea*. *Phytopathology 72*(6), 676-679.

Cho, M. S., Kang, M. J., & Kim, C. K. (2011). Sensitive and specific detection of *Xanthomonas oryzae* pv. oryzae by real-time bio-PCR using pathovar-specific primers based on an rhs family gene. *Plant Disease, 95*(5), 589-594.

Collado-Romero, M., Berbegal, M., & Jimenez-Diaz, R. M. (2009). A PCR-based 'molecular tool box'for in planta differential detection of *Verticillium dahliae* vegetative compatibility groups infecting artichoke. *Plant Pathology, 58*(3), 515-526.

Cooper, R. M., Flood, J., & Resende, M. L. . (1995). *The role of tannins and phytoalexins in the resistance of cocoa (Theobroma cacao L.) to Verticillium dahliae*. Paper presented at the Aspects of Applied Biology.

Dahm, R. (2008). Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. *Human Genetics, 122*(6), 565-581.

Dan, H., Ali-Khan, S. T., & Robb, J. (2001). Use of quantitative PCR diagnostics to identify tolerance and resistance to *Verticillium dahliae* in potato. *Plant Disease, 85*(7), 700-705.

Davis, J. R., Huisman, O. C., & Westermann, D. T. (1996). Effects of green manures on Verticillium wilt of potato. *Phytopathology, 86*(5), 444-453.

Davis, J. R., Pavek, J. J., & Corsini, D. L. . (1994). Influence of continuous cropping of several potato clones on the epidemiology of Verticillium wilt of potato. *Phytopathology, 84*(2), 207-214.

Davis, J. R., Stark, J. C., & Sorenesn, L. H. (1994). Interactive effects of nitrogen and phosphorus on Verticillium wilt of Russet Burbank potato. *American Potato Journal, 71*(7), 467-481.

Debode, J., Van Poucke, K., França, S. C., Maes, M., Höfte, Monica, & Heungens, K. (2011). Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. *Plant Disease, 95*(12), 1571-1580.

Demeke, T. , & Adams, R. P. (1992). The effects of plant polysaccharides and buffer additives on PCR. *Biotechniques, 12*(3), 332-334.

DeTrana, N. R. (2007). *Development of a Real-time PCR Assay for Detection and Quantification of Escherichia coli O157:H7 in Apple Juice.* (doctoral), University of Tennessee – Knoxville. /z-wcorg/ database. (oai:trace.tennessee.edu:utk_graddiss-1195)

Dhingra, O. D., & Sinclair, J. B. (1985). *Basic Plant Pathology Methods*: CRC Press, Inc.; Boca Raton, Florida; USA.

Duressa, D., Rauscher, G., & Koike, S. T. (2012). A real-time PCR assay for detection and quantification of *Verticillium dahliae* in spinach seed. *Phytopathology, 102*(4), 443-451.

Dwinell, L. D. (1967). *Selected Interactions of Verticillium dahliae, Pratylenchus penetrans, Major Nutrient Elements, Acer saccharum and Ulmus americana.* (Doctoral dissertation), Cornell University, Ithaca, N.Y.

El-Zik, K. M. (1985). Integrated control of Verticillium wilt of cotton. *Plant Disease 69*(12), 1025-1032.

Elmer, W. H., & Ferrandino, F. J. (2009). Suppression of Verticillium wilt of eggplant by earthworms. *Plant Disease, 93*(5), 485-489.

Emechebe, A. M., Leakey, C. L. A., & Banage, W. B. (1975). Verticillium wilt of cacao in Uganda: incidence and progress of infection in relation to time. *East African Agricultural and Forestry Journal, 41*(2), 184-186.

Engelbrecht, C., & Gleason, M. L. (2006). *Verticillium Wilt of Woody Plants*. Ames, Iowa: Iowa State University, University Extension.

Epstein, L., Beede, R., Kaur, S., & Ferguson, L. (2004). Rootstock effects on pistachio trees grown in *Verticillium dahliae*-infested soil. *Phytopathology, 94*(4), 388-395.

Erlich, H. A. . (1989). Polymerase chain reaction. *Journal of Clinical Immunology, 9*(6), 437-447.

Fahima, T., & Henis, Y. (1990). *Interactions between pathogen, host and biocontrol agent: multiplication of Trichoderma hamatum and Talaromyces flavus on roots of diseased and healthy hosts.* Paper presented at the Biological control of soil-borne plant pathogens.,

Department of Plant Pathology and Microbiology, Hebrew University of Jerusalem, POB 12, Rehovot, Israel.

Fradin, E. F., & Thomma, B. P. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology, 7*(2), 71-86.

Fradin, E. F., Zhang, Z., Ayala, J. C. J., Castroverde, C., Nazar, R. N., Robb, J., . . . Thomma, B. P. (2009). Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. *Plant Physiology, 150*(1), 320-332.

Frey, B., & Suppmann, B. (1995). Demonstration of the Expand™ PCR system's greater fidelity and higher yields with a lacI-based PCR fidelity assay. *Biochemica, 2*, 8-9.

Gayoso, C., de la Ilarduya, O. M., & Pomar, F. (2007). Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different Solanaceae cultivars. *European Journal of Plant Pathology, 118*(3), 199-209.

Gerik, J. S., Lommel, S. A., & Huisman, O. C. . (1987). A specific serological staining procedure for *Verticillium dahliae* in cotton root tissue. *Phytopathology, 77*(2), 261-265.

Gerstl, Z., Mingelgrin, U., & J., Krikun. (1977). Behavior and effectiveness of Vapam applied to soil in irrigation water. *Agricultural Research Organization Volcani Center Special Publication, 82*, 42-50.

Gilad, Z., & Borochov, A. (1993). Hot-water treatment of liatris tubers. *Scientia Horticulturae, 56*(1), 61-69.

Goud, J. K. , Termorshuizen, A. J., & Gams, W. (2003). Morphology of *Verticillium dahlia*e and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. *Mycological Research, 107*(7), 822-830.

Green, M. J., Thompson, D. A., & MacKenzie, D. J. (1999). Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease, 83*(5), 482-485. doi: 10.1094/pdis.1999.83.5.482

Hall, R., & Ly, H. (1972). Development and quantitative measurement of microsclerotia of *Verticillium dahliae*. *Canadian Journal of Botany, 50*(11), 2097-2102.

Hamelin, R. C., Bourassa, M., Rail, J., Dusabenyagasani, M., Jacobi, V., & Laflamme, G. (2000). PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderris canker of pine. *Mycological Research, 104*(5), 527-532.

Hayes, R. J., Vallad, G. E., & Qin, Q. (2007). Variation for resistance to Verticillium wilt in lettuce (*Lactuca sativa L.*). *Plant Disease, 91*(4), 439-445.

Hernández, M. E. , Davila, A. R., de Algaba, A. P., Lopez, M. A., & Casas, A. T. (1998). Occurrence and etiology of death of young olive trees in southern Spain. *European Journal of Plant Pathology, 104*(4), 347-357.

Hiemstra, J. A. (1995). *Verticillium Wilt of Fraxinus Excelsior.* (Ph.D thesis), Wageningen Agricultural University,, Ponsen & Looijen, Wageningen.

Hiemstra, J. A. , & Harris, D. C. (1998). *A Compendium of Verticillium Wilts in Tree Species*. Wageningen : CPRO-DLO: Ponsen & Looijen [for the Commission of the European Communities].

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology, 11*(9), 1026-1030.

Hoitink, H. A. J., Sydnor, T. D., & Wilson, C. L. (1979). Resistance of maple cultivars and species to Verticillium wilt - a preliminary report. *Research Circular, Ohio Agricultural Research and Development Center*(246), 46-47.

Holland, P. M., Abramson, R. D. , Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3'exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences, 88*(16), 7276-7280.

Hu, X., Bai, Y., Chen, T., Hu, D., & Yang, J. (2013). An optimized method for in vitro production of *Verticillium dahliae* microsclerotia. *European Journal of Plant Pathology, 136*(2), 225-229

Huisman, O. C. (1982). Interrelations of root growth dynamics to epidemiology of root-invading fungi. *Annual Review of Phytopathology, 20*(1), 303-327.

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., & Subbarao, K. V. (2011a). Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PloS One*, *6*(12), 28341.

Inderbitzin, Patrik, Bostock, Richard M, Davis, R Michael, Usami, Toshiyuki, Platt, Harold W, & Subbarao, Krishna V. (2011b). Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. *PloS one*, *6*(12), e28341.

Isaac, I. (1949). A comparative study of pathogenic isolates of *Verticillium*. *Transactions of the British Mycological Society*, *32*(2), 137-157.

Isaac, I., Fletcher, P., & Harrison, J. A. C. (1971). Quantitative isolation of *Verticillium* spp. from soil and moribund potato haulm. *Annals of Applied Biology*, *67*(2), 177-183.

Isaac, Ivor. (1967). Speciation in *Verticillium*. *Annual Review of Phytopathology*, *5*(1), 201-222.

Jimenez-Diaz, R. M., Cirulli, M., Bubici, G., del Mar Jimenez-Gasco, M., Antoniou, P. P., & Tjamos, E. C. (2012). Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. *Plant Disease*, *96*(3), 304-329.

Jordan, V. W. L. (1971). Estimation of the distribution of *Verticillium* populations in infected strawberry plants and soil. *Plant Pathology*, *20*(1), 21-24.

Karajeh, M. (2006). Seed transmission of *Verticillum dahlia*e in olive as detected by a highly sensitive nested PCR-based assay. *Phytopathologia Mediterranea, 45*(1), 15-23.

Karajeh, M. R., & Masoud, S. A. (2006). Molecular detection of *Verticillium dahliae* Kleb. in asymptomatic olive trees. *Journal of Phytopathology, 154*(7-8), 496-499.

Katan, J., Greenberger, A., Alon, H., & Grinstein, A. (1976). Solar heating by polyethylene mulching for the control of diseases caused by soil-borne pathogens. *Phytopathology, 66*(5), 683-688

Klingner, A. E., C., Hildebra. D., & Wilhelm, S. (1971). Occurrence of *Erwinia carotovora* in the rhizosphere of cotton plants which escape verticillium wilt. *Plant and Soil, 34*(1), 215-218.

Klosterman, S. J. , Atallah, Z. K., Subbarao, K. V., & Vallad, G. E. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology, 47*, 39-62 doi: 10.1146/annurev-phyto-080508-081748

Koike, S. T., & Subbarao, K. V. (2000). Broccoli residues can control Verticillium wilt of cauliflower. *California Agriculture, 54*(3), 30-33.

Kramer, M. F., & Coen, D. M. (2002). The Polymerase Chain Reaction. *Current Protocols in Protein Science*, A. 4J. 1-A. 4J. 8. doi: 10.1002/0471140864.psa04js29

Langcake, P. (1981). Alternative Chemical Agents for Controlling Plant Disease *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 295*(1076), 83-101.

Larkin, R. P., Honeycutt, C. W., & Olanya, O. M. (2011). Management of Verticillium wilt of potato with disease-suppressive green manures and as affected by previous cropping history. *Plant Disease, 95*(5), 568-576.

Leben, S. D., Wadi, J. A., & Easton, G. D. (1987). Effects of *Pseudomonas fluorescens* on potato plant growth and control of *Verticillium dahliae*. *Phytopathology, 77*(11), 1592-1395. doi: 10.1094/Phyto-77-1592

Li, K. N., Rouse, D. I., & German, T. L. (1994). PCR primers that allow intergeneric differentiation of ascomycetes and their application to *Verticillium* spp. *Applied and Environmental Microbiology, 60*(12), 4324-4331.

Li, R., Mock, R., Huang, Q., Abad, J., Hartung, J., & Kinard, G. (2008). A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. *Journal of Virological Methods, 154*(1–2), 48-55.

Lopez-Escudero, F. J., & Blanco-Lopez, M. A. (2001). Effect of a single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. *Plant Disease, 85*(5), 489-496.

Lopez-Escudero, F. J., Del Rio, C., Caballero, J. M., & Blanco-Lopez, M. A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. *European Journal of Plant Pathology, 110*(1), 79-85.

Lopez, M. M., Bertolini, E., Olmos, A., Caruso, P., Gorris, M. T., Llop, P., . . . Cambra, M. (2003). Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology*, *6*(4), 233-243.

Lumsden, R. D. (1981). A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. *Phytopathology, 71*(3), 282-285. doi: 10.1094/Phyto-71-282

Mace, M. E., Bell, A. A., & Beckman, C. H. (1981). *Fungal Wilt Diseases of Plants*: Academic Press, Inc: New York, N.Y., USA; London, England. Iillus.

MacKenzie, D. J., McLean, M. A., Mukerji, S., & Green, M. J. (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease*, *81*(2), 222-226.

Martin, R. R., James, D., & Levesque, C. A. (2000). Impacts of Molecular Diagnostic Technologies on Plant Disease Management *Annual Review of Phytopathology* (Vol. 38, pp. 207-239).

Maruthachalam, K., Atallah, Z. K., Vallad, G. E., Klosterman, S. J., Hayes, R. J., Davis, R. M., & Subbarao, K. V. (2010). Molecular variation among

isolates of *Verticillium dahliae* and polymerase chain reaction-based differentiation of races. *Phytopathology*, *100*(11), 1222-1230.

Mascarello, N., Favaron, F., & Di Lenna, P. (2001). Evaluation of a PCR-based method to detect *Verticillium dahliae* in chicory taproots. *Journal of Plant Pathology*, *83*(3), 221-224.

McCartney, H. A., Foster, S. J., Fraaije, B. A., & Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Science, 59*(2), 129-142.

McGuire, A. M. (2003). Mustard green manures replace fumigant and improve infiltration in potato cropping system. *Crop Management*, *22*(August), 1-6

Mercado-Blanco, J., Rodriguez-Jurado, D., Parrilla-Araujo, S., & Jimenez-Diaz, R. M. (2003). Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Plant Disease, 87*(12), 1487-1494.

Mol, L. (1995). *Agronomic Studies on the Population Dynamics of Verticillium dahliae.* (doctoral), Landbouwuniveristeit te Wageningen, Wageningen, The Netherlands. /z-wcorg/ database. (1959)

Moorman, G. W. (1982). The influence of black plastic mulching on infection rates of Verticillium wilt and yield of eggplant. *Phytopathology, 72*(11), 1412-1414. doi: 10.1094/Phyto-72-1412

Mumford, R. A., Walsh, K., Barker, I., & Boonham, N. (2000). Detection of Potato mop top virus and Tobacco rattle virus using a multiplex real-time

fluorescent reverse-transcription polymerase chain reaction assay. *Phytopathology*, *90*(5), 448-453. doi: 10.1094/PHYTO.2000.90.5.448

Neumann, M. J., & Dobinson, K. F. (2003). Sequence tag analysis of gene expression during pathogenic growth and microsclerotia development in the vascular wilt pathogen *Verticillium dahliae*. *Fungal Genetics and Biology, 38*(1), 54-62. doi: 10.1016/S1087-1845(02)00507-8

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136. doi: 10.1016/j.jviromet.2005.11.005

Parker, K. G. (1959). *Verticillium hadromycosis* of deciduous tree fruits. *Plant Disease Reporter 255*(Suppl), 39-61 pp.

Parkhi, V., & Kumar, V. (2010). Expression of arabidopsis NPR1 in transgenic cotton confers resistance to non-defoliating isolates of *Verticillium dahliae* but not the defoliating isolates. *Journal of Phytopathology, 158*(11-12), 822-825.

Pasche, J. S., Mallik, I., Anderson, N. R., & Gudmestad, N. C. (2013). Development and validation of a real-time PCR assay for the quantification of *Verticillium dahliae* in potato. *Plant Disease, 97*(5), 608-618. doi: 10.1094/PDIS-06-12-0554-RE

Pasche, J. S., Thompson, A. L., & Gudmestad, N. C. (2013). Quantification of field resistance to *Verticillium dahliae* in eight russet-skinned potato

cultivars using real-time PCR. *American Journal of Potato Research, 90*(2), 158-170.

Pegg, G. F., & Brady, B. L. (2002). *Verticillium Wilts* CABI Publishing, 10 E. 40th Street, Suite 3203, New York, NY, 10016, USA.

Pernezny, K., Roberts, P. D., & Goldberg, N. (2003). Verticillium wilt. In K. Pernezny, P. D. Roberts & J. F. Murphy (Eds.), *Compendium of Pepper diseases* (pp. 21-22). Everglades Research and Education Center, University of Florida, Belle Glade, Florida, USA.

Picman, A. K., Schneider, E. F., & Gershenzon, J. (1990). Antifungal activities of sunflower terpenoids. *Biochemical Systematics and Ecology, 18*(5), 325-328. doi: 10.1016/0305-1978(90)90005-Z

Picman, AK, & Schneider, EF. (1990). Antifungal activities of sunflower terpenoids. *Biochemical Systematics and Ecology*, *18*(5), 325-328.

Pirone, P. P., Dodge, B. O., & Rickett, H. W. (1960). *Diseases and Pests of Ornamental Plants*: New York, The Ronald Press Co.

Pirone, Pascal P. (1978). *Diseases and Pests of Ornamental Plants*. New York; USA: John Wiley & Sons.; New York; USA.

Piskur, B., Ogris, N., & Jurc, D. (2007). Species-specific primers for *Eutypella parasitica,* the causal agent of Eutypella canker of maple. *Plant Disease, 91*(12), 1579-1584. doi: 10.1094/PDIS-91-12-1579

Plasencia, J., & Banttari, E. E. (1997). Comparison between a culture plate method and an immunoassay to evaluate vascular colonization of potato

by *Verticillium dahliae*. *Plant Disease, 81*(1), 53-56. doi: 10.1094/PDIS.1997.81.1.53

Powell, N. T. (1971). Interactions between nematodes and fungi in disease complexes. *Annual Review of Phytopathology, 9*(1), 253-274. doi: 10.1146/annurev.py.09.090171.001345

Pullman, G. S., DeVay, J. E., & Garber, R. H. (1981). Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. *Phytopathology, 71*(9), 959-964.

Ramsfield, T. D., Dobbie, K., Dick, M. A., & Ball, R. D. (2008). Polymerase chain reaction-based detection of *Fusarium circinatum*, the causal agent of pitch canker disease. *Molecular Ecology Resources, 8*(6), 1270-1273.

Rasmussen, R. (2001). Quantification on the LightCycler. In P. D. m. S. Meuer, P. D. C. Wittwer & D. K.-I. Nakagawara (Eds.), *Rapid Cycle Real-Time PCR* (pp. 21-34): Springer Berlin Heidelberg.

Rowe, R. C., & Powelson, M. L. (2002). Potato early dying: management challenges in a changing production environment. *Plant Disease, 86*(11), 1184-1193.

Rubin, B., & Benjamin, A. (1984). Solar heating of the soil: involvement of environmental factors in the weed control process. *Weed Science, 32*(1), 138-142.

Rudolph, Bert Alexander. (1929). *Verticillium hadromycosis*: Department of Botany, Stanford University.

Russell, E. J. (1920). The partial sterilization of soils. *Journal of the Royal Horticultural Society, 45*(2-3), 237-246.

Schnathorst, W. C. (1981). Life cycle and epidemiology of *Verticillium Fungal Wilt Diseases of Plants* (pp. XIV+640P): Acadimac Press, Inc.: New York, N.Y., USA.

Schoch, C. L., Sung, G., López-Giráldez, F., Townsend, J. P., Miadlikowska, J. , & Hofstetter, V. (2009). The Ascomycota tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Systematic Biology, 58*(2), 224-239.

Sezgin, E. (1982). Investigations on the effects of some cultural applications and antagonistic fungi on Rhizoctonia solani Kuhn. and Verticillium dahliae Kleb. in the Aegean region. I. Effects of crop rotation and fertilizations. *Journal of Turkish Phytopathology, 11*(1-2), 41-54.

Shanmugam, N., Vinayagamurthy, A., Rajendran, G., Muthukrishnan, T. S., & Kandaswamy, T. K. (1977). Occurrence of nematode-fungus interactions on cotton around Coimbatore. *Science and Culture, 43*(8), 346-348.

Sinclair, W. A., & Lyon, H. H. (2005). *Diseases of Trees and Shrubs*: Comstock Publishing Associates.

Smith, K. L. (1976). *Verticillium Wilt of Maples: Stem Colonization by the Pathogen and Prospects for Chemotherapy.* Cornell University. Available from WorldCat

Soesanto, L. (2000). *Ecology and Biological Control of Verticillium dahliae.* (Ph.D), Wageningen University, Wageningen, Netherlands., Landbouwuniversiteit Wageningen. /z-wcorg/ database.

Stapleton, J. J., Paplomatas, E. J., Wakeman, R. J., & Devay, J. E. (1993). Establishment of apricot and almond trees using soil mulching with transparent (solarization) and black polyethylene film: effects on Verticillium wilt and tree health. *Plant Pathology, 42*(3), 333-338. doi: 10.1111/j.1365-3059.1993.tb01509.x

Sundaram, S., Plasencia, J., & Banttari, E. E. (1991). Enzyme-linked immunosorbent assay for detection of *Verticillium* spp. using antisera produced to *V. dahliae* from potato. *Phytopathology, 81*(12), 1485-1489. doi: 10.1094/Phyto-81-1485

Tabaeizadeh, Z., Agharbaoui, Z., Harrak, H., & Poysa, V. (1999). Transgenic tomato plants expressing a *Lycopersicon chilense* chitinase gene demonstrate improved resistance to *Verticillium dahliae* race 2. *Plant Cell Reports, 19*(2), 197-202.

Tenuta, M., Conn, K. L., & Lazarovits, G. (2002). Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. *Phytopathology, 92*(5), 548-552. doi: 10.1094/PHYTO.2002.92.5.548

Tenuta, M., & Lazarovits, G. (2002). Ammonia and nitrous acid from nitrogenous amendments kill the microsclerotia of *Verticillium dahliae*. *Phytopathology, 92*(3), 255-264.

Termorshuizen, A. J., Davis, J. R., Gort, G., Harris, D. C., & Huisman, O. C. (1998). Interlaboratory comparison of methods to quantify microsclerotia of *Verticillium dahliae* in soil. *Applied and Environmental Microbiology, 65*(6), 2805-2805

Tjamos, E. C., Rowe, R. C., Heale, J. B., & Fravel, D. R. (2000). *Advances in Verticillium: research and disease management*, St. Paul; USA.

Typas, M. A., & Heale, J. B. (1976). Acriflavine-induced hyaline variants of *Verticillium albo-atrum* and *V. dahliae*. *Transactions of the British Mycological Society, 66*(1), 15-25.

Usami, T. , Ishigaki, S., Takashina, H., Matsubara, Y., & Amemiya, Y. (2007). Cloning of DNA fragments specific to the pathotype and race of *Verticillium dahliae*. *Journal of General Plant Pathology, 73*(2), 89-95. doi: 10.1007/s10327-006-0334-4

Van de Koppel, M. M., & Schots, A. (1995). Monoclonal antibody-based double-antibody sandwich-ELISA for detection of *Verticillium* spp. in ornamentals. *Phytopathology, 85*(5), 608-612.

Van Loenen, M. C. A., Turbett, Y., Mullins, C. E., Feilden, N. E. H., Wilson, M. J., Leifert, C., & Seel, W. E. . (2003). Low temperature–short duration steaming of soil kills soil-borne pathogens, nematode pests and weeds. *European Journal of Plant Pathology, 109*(9), 993-1002. doi: 10.1023/B:EJPP.0000003830.49949.34

Walker, Nigel J. (2002). A technique whose time has come. *Science, 296*(5567), 557-559.

Wang, Y. L., Wang, Y., & Tian, C. M. (2013). Quantitative detection of pathogen DNA of Verticillium wilt on smoke tree *Cotinus coggygria*. *Plant Disease, 97*(12), 1645-1651 doi: 10.1094/PDIS-04-13-0406-RE

Watson, J. D., & Crick, F. H. C. (1953). A structure for deoxyribose nucleic acid. *Nature, 171*(3), 737-738.

Weiland, J. E., Littke, W. R., & Haase, D. L. (2013). Forest nurseries face critical choices with the loss of methyl bromide fumigation. *California Agriculture, 67*(3), 153-161

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics* (Vol. 18). Hoffmann-LA Roche, Emeryville, Calif 94608, USA: Academi Press, Inc.: San Diego California, USA; London, England, UK. Iillus.

Wiggins, B. E., & Kinkel, L. L. (2005). Green manures and crop sequences influence potato diseases and pathogen inhibitory activity of indigenous streptomycetes. *Phytopathology, 95*(2), 178-185. doi: 10.1094/PHYTO-95-0178

Witzell, J., & Martin, J. A. (2008). Phenolic metabolites in the resistance of northern forest trees to pathogens - past experiences and future prospects. *Canadian Journal of Forest Research, 38*(11), 2711-2727. doi: 10.1139/X08-112

Yucel, F., Benlioglu, S., Basalp, A., Ozturk, S., & Benlioglu, K. (2005). Development of a double monoclonal antibody sandwich ELISA test for *Verticillium dahliae* Kleb. *Food and Agricultural Immunology*, *16*(04), 283-291. doi: 10.1080/09540100500399700

Zare, R., Gams, W., & Hans-Josef, S. (2004). The type species of *Verticillium* is not congeneric with the plant-pathogenic species placed in *Verticillium* and it is not the anamorph of 'Nectria'inventa. *Mycological Research, 108*(5), 576-582.

Zhang, Y. P., Uyemoto, J. K., & Kirkpatrick, B. C. (1998). A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *Journal of Virological Methods, 71*(1), 45-50. doi: 10.1016/S0166-0934(97)00190-0

Zheng, G. G. , Chen, R. H., Wang, Y. C., Wang, K. R., & Zheng, X. B. (2005). Molecular Detection of *Verticillium albo-atrum* by PCR Based on Its Sequences. *Agricultural Sciences in China, 4*(10), 760-766.

Zimm, L. A. (1918). A wilt disease of maples. *Phytopathology, 8*, 80-81.

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EDUCATION

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PROFESSIONAL POSITIONS HELD

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University of Al_Qadisiya, Agriculture College

Laboratory Technician, 2008-2011

HONORS

Honor on 17/03/2009 under No. 415 from Agricultural College, University of AL_Qadisiya, based on my scientific activities in University of Al_Qadisiya, Agriculture College, I have got the honor from the Dean of the College.

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PROFESSIONAL PRESENTATIONS

Oral Presentations

Polymerase Chain Reaction Inhibition in Environmental Samples, Departmental Seminar at the University of Kentucky, Department of Plant Pathology, September 16, 2013

Development of an Assay for Rapid Detection and Monitoring of *Verticillium dahliae* in Woody Plants by Real-time Polymerase Chain Reaction (PCR), 2014 American Phytopathology Association (APS) Southern Division Meeting, February 2-3, 2014, in Dallas, Texas

Evaluation of PCR-Based Methods for Rapid Detection and Monitoring of *Verticillium dahliae* in Woody Hosts by Real-Time Polymerase Chain Reaction, Thesis Seminar, University of Kentucky Department of Plant Pathology, April 1, 2014

PROFESSIONAL PUBLICATIONS

Media Interviews

An article, UK researcher finds molecular markers for common woody plant disease, on University of Kentucky College of Agriculture, Food and Environment News Website by Katie Pratt, January 21, 2014

Abstracts

Aljawasim, B. D. G., Vincelli, P. (2014). Development of an Assay for Rapid Detection and Monitoring of *Verticillium dahliae* in Woody Plants by Real-time Polymerase Chain Reaction (PCR). 2014 APS Southern Division Meeting, February 2-3, 2014

WORKSHOPS

I participated in Real-Time PCR workshop in the Department of Plant Pathology, at the University of Kentucky College of Agriculture, Food and Environment, January 28, 2013